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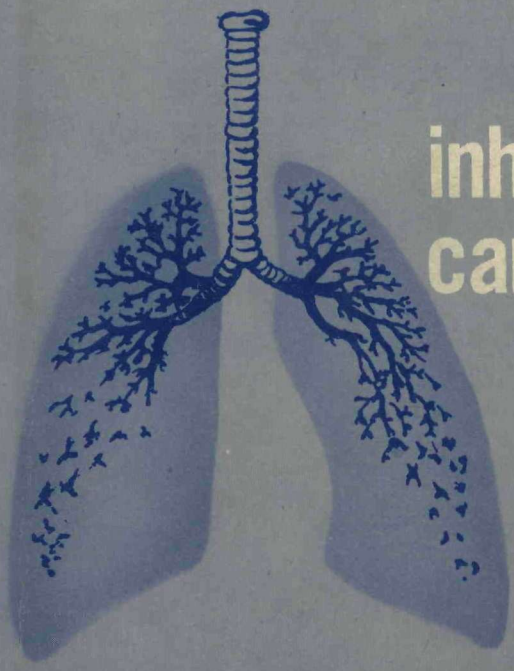

inhalation carcinogenesis

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inhalation carcinogenesis

Proceedings of a Biology Division, Oak Ridge National Laboratory, conference held in Gatlinburg, Tennessee, October 8—11, 1969

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and
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Editors

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April 1970

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FOREWORD

The nuclear energy community has been concerned with the untoward sequelae of radioactive insults to the biosphere since the earliest days of the Manhattan Project. As for carcinogenesis by radioactive inhalants, the unhappy experiences of the Joachimsthal miners had been much in the thoughts of the nuclear community at the time the original radioactive inhalation experiments at Rochester were set up in the 1940's.

Radioactive insult is only one of many physical insults to the environment. It therefore seemed natural, especially as our society became more sensitive to the environment, and as our politicians became more aware of people's concern in this regard, that the techniques and logistic powers developed at the National Laboratories for dealing with radioactive insult be applied and extended to other physical insults. Out of this concern grew a program at the Oak Ridge National Laboratory, jointly funded by the National Cancer Institute and the Atomic Energy Commission, to study carcinogenesis (particularly inhalation carcinogenesis) by various physical inhalants, both singly and conjointly. This Gatlinburg conference is a natural step in bringing together the many workers in this difficult and demanding field.

It is difficult and demanding in part because one is trying to detect effects near thresholds. In so many of our attempts to assess biological effects of environmental pollutants we are concerned with effects at low levels. To put the question another way, we are trying to determine whether effects found at very high levels are linear down to very low levels.

Whether this is a fully soluble problem – whether we can ever hope to know if there is or is not a carcinogenic threshold for exposure to cigarette smoke, or radioactivity, or SO_2, or automobile exhaust – is an open question. Yet if one examines the arguments presented by the environmentalists (and all of us are environmentalists today to some degree), this is the scientific crux of the matter.

Such questions, if they are at all answerable, probably will require the style of big biology – big protocols, large epidemiologic studies, expensive logistics – in short, the style that characterizes the AEC's National Laboratories. I would therefore expect Oak Ridge to become more involved with these matters, including symposia such as this one, which aim at helping to establish a tolerable equilibrium between Man and his Technological Environment.

Alvin M. Weinberg
Oak Ridge National Laboratory*

*Operated by Union Carbide Corporation for the U.S. Atomic Energy Commission.

PREFACE

The deterioration of man's relationship with his environment is being documented by an increasing number of alarming findings; among them is a clear link between the induction of cancer and the air we breathe. A positive correlation exists between the increased rate of lung cancer in humans and environmental air pollutants – such as motor vehicle exhaust, coal and asphalt dusts, fumes from fuel oils, and cigarette smoke. It is equally clear that long and heavy occupational exposures to high concentrations of dusts containing uranium and other radionuclides, nickel, asbestos, or chromate particles also influence the incidence of lung cancer in man. Until recently these associations have been based primarily on retrospective and prospective population studies, but experimental evidence linking many of these suspected carcinogens to neoplasia in lower animals is increasing.

Although multidisciplinary studies in inhalation carcinogenesis have been relatively unrewarding as an experimental approach to environmental problems (most likely because of biologic and technologic complexities), they offer the most ideal simulation of human exposure to air pollutants.

One of the most important needs in the study of respiratory carcinogenesis in man is for a relevant model for testing suspected and known carcinogens. It is imperative that this model closely simulate the human mode of exposure so that bronchial mucosal changes and tumors of bronchial origin in the test animal are comparable to those found in man. This simulation should preferably be achieved with rodent species, since they offer many advantages as test animals. A second criterion is that the experimental technique lend itself to testing natural and chemical forms of the suspected and known carcinogens. Inhalation systems are potentially able to meet these requirements. As an added advantage, these systems can involve large numbers of animals in a single

experiment – an important point because of the low natural or induced incidence of lung cancer. Thus, it is time that we assess research in inhalation carcinogenesis to find how well it is filling this need for a relevant model.

The Conference on Inhalation Carcinogenesis was convened primarily to evaluate the importance and feasibility of using inhalation techniques to study respiratory carcinogenesis. (There is no doubt that many of the questions related to carcinogenesis, and more specifically to respiratory carcinogenesis, could be answered adequately by more scientifically manageable procedures.) At the same time, it was thought important to determine the status of chronic inhalation studies now underway, particularly those specifically designed to answer some of the important questions associated with respiratory carcinogenesis. As a result of this conference we hoped there would evolve a clearer understanding of inhalation experiments so that we may better judge their value as models for the human disease.

A considerable portion of the conference was devoted to the technology of inhalation studies. This emphasis seems warranted by the important role that improved equipment and procedures have played in the successful production of lung cancer in animal models, as shown by several studies in this conference. The results presented indicate that inhalation technology will occupy a vital spot in future studies.

In general, it was the aim of the sponsoring agencies – the National Cancer Institute and the U.S. Atomic Energy Commission – that a conference on inhalation carcinogenesis would place the results of inhalation studies in their proper perspective within the rapidly developing concern over environmental influences on man. This hope seems to have been realized, as the findings presented in these proceedings demonstrate. We also feel that the discussions which follow the formal presentations produce an awareness of factors in experiments that will prove important for the final application of the basic principles of inhalation carcinogenesis to the field of respiratory cancer.

The final session of the conference was concerned with program planning in inhalation carcinogenesis. Drs. Carl Baker and Umberto Saffiotti, representing the National Cancer Institute; Dr. C. R. Richmond, representing the Atomic Energy Commission; and Dr. W. W. Payne, representing the

National Institute of Environmental Health Sciences, each presented his agency's present program and future plans concerning respiratory carcinogenesis. The results of this session demonstrate that these three organizations have a commitment to investigate the rapidly increasing number of chemical, physical, and microbiological agents in the environment; to obtain meaningful information on realistic concentrations of these agents, either singly or in combination; and then to relate these data to respiratory carcinogenesis.

The conference was sponsored by the National Cancer Institute and the U.S. Atomic Energy Commission and organized by the Biology Division of the Oak Ridge National Laboratory, with the help of Drs. Umberto Saffiotti and C. R. Richmond. We were fortunate to have Drs. J. L. Liverman, D. G. Doherty, Clayton Loosli, T. T. Crocker, Norton Nelson, and Carl G. Baker as session chairmen.

Finally, we are indebted to a large number of individuals and organizations for their help in ensuring the smooth operation of the conference and the rapid publication of the proceedings. Special credit should go to the Technical Information personnel, Personnel Services, and Instrumentation and Controls Division of ORNL; to the Editorial Office and administrative staff of the Biology Division; and to the Editorial, Graphic Arts, and Printing Branches, and Life Sciences Section, of the AEC Division of Technical Information Extension. Much of the editorial burden was skillfully handled by Marian Harrison and Nancy Trent, and we gratefully acknowledge their assistance.

We are especially grateful to Drs. J. L. Liverman and F. T. Kenney for their beneficial counsel during all stages of the conference.

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P. Nettesheim
J. R. Gilbert
Biology Division
Oak Ridge National Laboratory

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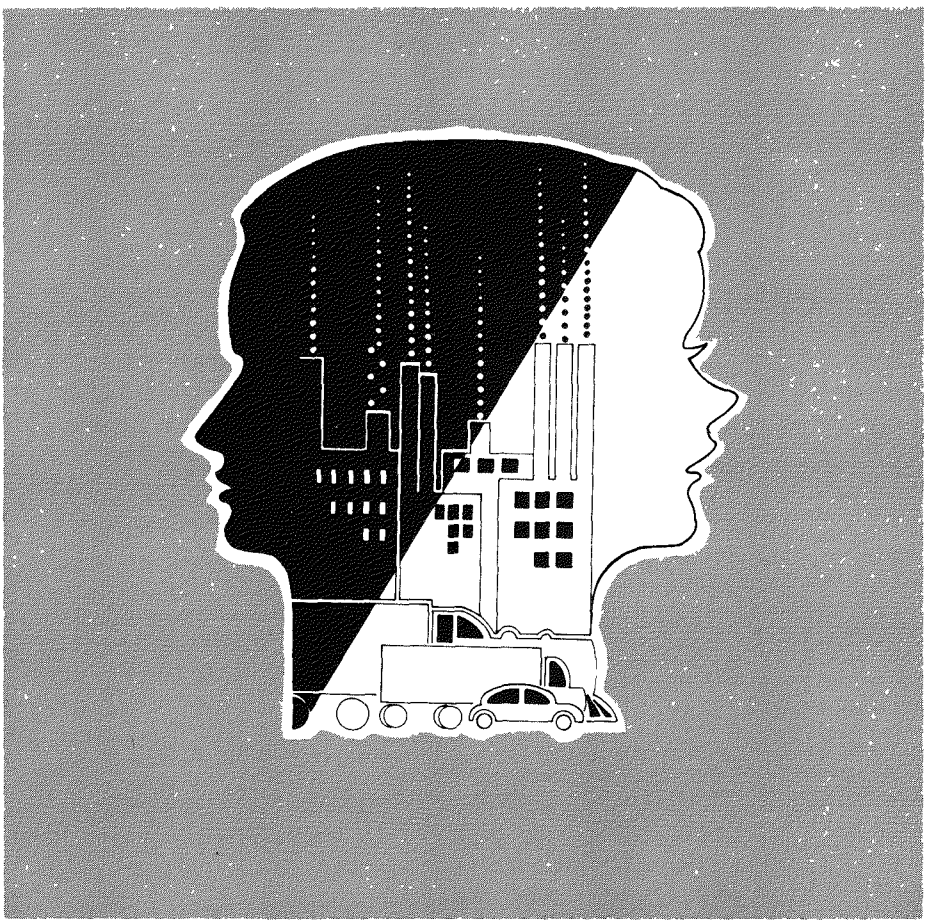
Chairman – J. L. Liverman
Director's Division
Oak Ridge National Laboratory
Oak Ridge, Tennessee



INHALATION CARCINOGENESIS IN MAN: ENVIRONMENTAL AND OCCUPATIONAL HAZARDS

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ABSTRACT

There has now accumulated extensive evidence on the induction of lung cancer via inhalation in man The best documented and most decisive evidence relates to occupational exposures Examples would include radon daughters, chromate chemical manufacturing, asbestos exposure There is also evidence suggesting that the generally lower levels of exposures found in community air may be associated with an increased risk of lung cancer Selected examples of such associations will be examined and discussed, and the relationship of laboratory studies to such problems will be considered

Lung cancer has been for some decades and remains at this time a major health problem in this and many other countries, in this country it currently accounts for some 60,000 deaths per year We now know beyond any reasonable doubt that cigarette smoking is a major contributor to this problem However, when the day arrives, as it perhaps will, when cigarette smoking is a negligible source of lung cancer, it seems probable that environmentally induced lung cancer will remain a problem of substantial importance

There are now many examples, some strikingly unequivocal, others only suggestive, wherein lung cancer in man arises from environmental factors At the present time, it is probably safe to say that something of the order of 95% of all lung cancer is of environmental origin. Currently the bulk of this percentage has its source in cigarette smoking, it seems probable that of the residue not attributable to cigarette smoking a major part also arises from environmental sources In short, lung cancer seems to be predominantly of environmental origin. Indeed, the lung (with the skin as a possible contender) now may head the list of human organs for the variety and number of instances in which environmental agents are involved in cancer induction That the lung should share this role with skin reflects their direct contact with environmental agents

and the limitations in the mechanisms they have for protection against such noxious factors

The benefits deriving from the accessibility of reliable laboratory models has been well demonstrated in the control of bladder cancer arising from aromatic amines and in skin cancer, particularly from polynuclear hydrocarbons In each instance protective measures have led to very substantial gains One can never know when new processes are introduced what would have been the consequences had not preventive measures been instituted, but it seems certain that there would have been a very large toll of occupational skin cancer had not the laboratory evaluation of the carcinogenicity of certain petroleum products and fractions led to the careful control of those identified as hazardous In a similar way laboratory models are indispensable for the institution of rational preventive measures for lung cancer However, we are still in a relatively primitive state at this time with respect to predictive testing for lung cancer hazards

I would like to review with you evidence relating environmental agents to lung cancer in man and, in the course of the review, take note of the experimental work bearing on these examples It will develop, I believe, that the laboratory has served a very important, though still inadequate, role

Laboratory studies can fulfill a variety of ends in aiding us in the human problem, among these are (1) a safety evaluation aimed at determining whether an agent is a lung cancer hazard for man, (2) pinpointing the active agent or agents among a number rendered suspect by epidemiological study, (3) estimating dose response relationships, or, more realistically, determining dose-response *patterns* (rather than absolute values), (4) developing a better understanding of the biological course of the disease, and (5) improving diagnostic detection

Of these ends the first is generally the most difficult The course of toxicological appraisal of human health hazards is strewn with experiments which were regarded (often naively) as faithfully simulating human exposure, but which failed to do so A reliable and truly faithful simulation of human exposure can be very complex and difficult to achieve, and a failure can, of course, be very misleading In the example to be discussed immediately there were at least two such failures [1 2]

What is probably the most ancient and most well-established example of environmental lung cancer is still with us, and is still in need of study, I refer to lung cancer from radioactive radon daughter products The miners' disease observed in the Middle Ages in the mines of Joachimsthal and Schneeberg was identified as lung cancer in the 1879's There is no doubt that we currently have the identical problem in the uranium mines on our Colorado plateau.

It may be informative to review in some detail the development of our understanding of this exposure-disease pattern Originally, the mines were worked, of course, for materials other than uranium, but uranium was there, as were the radon daughters Speculation as to the cause of the disease included

arsenic and nickel In the 1920's a suggestion was made that radioactive materials were involved It was, however, generally assumed that radon gas itself was the responsible agent (see Pirchan and Sikl[2] for the status in 1932) With further study, it became apparent that the bronchial epithelium was the target tissue With this closer identification, it developed that dosage from the radon daughter products would exceed that from the radon itself This was quantitatively assessed in a classic study by Altshuler [3]

It will be useful to briefly review the task which Altshuler undertook in this examination of radiation dosage to the lung, since it illustrates well a series of dosimetric factors for the lung which will have relevance elsewhere

The lung is a relatively complex organ whose anatomical, functional, and histological characteristics determine the vulnerability of the lung to inhaled noxious agents The mine atmosphere, in turn, is a complex entity in which radionuclides coming from radon have differing physical characteristics – ranging from the gas and the radon, to metallic daughter products of radon, some of which may exist as extremely small particulates composed of a relatively small number of metallic atoms, to other daughter products, which having become attached to dust of all sizes in the mine atmosphere take on the aerodynamic characteristics of these particles The aerodynamic parameters (size, shape, and density) of this entire range of particulates determine the extent and locus deposition in the lung The radiological characteristics (half-life and tissue penetration of the alpha particles from the radon daughters) modify dosage to the basal cells of epithelium The lung characteristics (size and geometry of the various airway branchings) control deposition patterns The rate of movement of the mucous sheet controls the time of exposure of various parts of the bronchial epithelium, while the combined thickness of the epithelium and mucous sheet alter the effectiveness of alpha irradiation of the basal cells by radionuclides lying on the sheet

Altshuler undertook a quantitative analysis of the dosimetry of the various parts of the lung by the analysis of these factors in a dynamic model Of particular interest was (1) the prediction that the heaviest dosage would be delivered to the epithelium of the segmental bronchi, the observed probable site of origin of the majority of miners' tumors, and (2) an estimate of dose delivered by one "working level" (WL) The dosage from one WL over a 1-year period, for 8 hr per day and a 5-day week, was estimated at 33 rads We do not yet know what the intrinsic radiation sensitivity for the production of cancer in epithelial cells is for alpha irradiation, although we are attempting to make such estimates We do have good quantitative information for beta irradiation of the bronchial epithelium [4] If we make the assumption that the relative biological effectiveness of alpha radiation for the bronchial epithelium is similar to the value of 3 (for single acute exposure) found[5] for the epithelium of the skin, one can derive from Altshuler's work an estimate of total dose in rems Such a computation leads to an estimated dose of 2000 rems for a 20-year occupational exposure A dose of this magnitude in rats (beta irradiation), in work done in this laboratory,

led to about 10% cancer incidence using an in-lying radioactive pellet [4] This is quite consonant with the epidemiological data on exposed miners Indeed, the agreement is probably closer than circumstances justify considering all the uncertainties in this kind of estimate A considerable uncertainty, for example, on the experimental side, is the unknown role of the irritation associated with the presence of the implanted pellet used as a carrier of the radioactive beta emitting ruthenium in these experiments It is even possible that this factor may have had a stimulatory role comparable to the synergism found between cigarette smoking and irradiation in the production of lung cancer in miners [6]

The epidemiological data on miners has been under some challenge because of the small numbers involved Although the upper part of the dose response curve is generally accepted, the lower part dealing with low incidence levels is predictably less decisive statistically because of the even smaller number of individuals in each dose group. Experimental work has provided us with data on the form of the dose response curve [4] The form of the experimental curve closely resembles that found epidemiologically and can reassure us on the pattern of the curve at these lower incidence levels, thus permitting us to use the human data with more confidence

For a variety of reasons experimental inhalation exposures adequately simulating mine exposures have not yet been conducted, but clearly need doing. Studies outlining more adequate simulation will be described at this meeting. However, inhalation studies yielding lung cancer have been made with plutonium[7] and polonium.[8]

This example has been presented in some detail since it illustrates a series of principles which are important in dealing with the practical problems of environmental lung cancer It also serves to illustrate how experimental inputs from a number of experimental approaches can contribute to a better definition of the human problem. Obviously, with inhaled chemical agents, there are additional factors such as tissue metabolism which will affect local tissue dosage. On the other hand, the interplay of physical characteristics of the inhaled material (gas or dust) with the lung characteristics will again be important dosimetric considerations. Also, conditioning factors and interaction (e.g., cigarette smoking) are to be anticipated as possibly important determinants

I would now like to touch more briefly on other sources of lung cancer One could spend the entire time available, and more, reviewing the extensive studies bearing on lung cancer from cigarette smoking. We all know that epidemiological evidence is now overwhelmingly persuasive, and although the laboratory work is clearly inadequate, it is not incompatible with epidemiological evidence. Nevertheless, the limited success in experimental confirmation has had two undesirable consequences, (1) it has provided a continuing, though weakening, defense on the part of those anxious to continue to smoke themselves or to see others smoke, (2) it has stood as a barrier to the systematic engineering of a safer cigarette.

Most of the inhalation studies on cigarette smoke so far have suffered from the lack of back-up of a valid laboratory model Failure was predictable in inhalation exposures of small numbers of rodents to concentrations of smoke far below that received by moderate to heavy smokers, among whom incidence levels were of the order of 3 per 1000, a cancer incidence not detectable in the usual laboratory tests If this were not enough, inhalation exposure to extremely high concentrations of polynuclear hydrocarbons alone produces no cancer, however, and significantly, joint exposure of lower concentrations of polynuclear hydrocarbons with an irritant does produce lung cancer [9] Although some studies that avoid the low dose problem are now under way, we do not yet have adequate mastery of our experimental models to carry out an inhalation study of cigarette smoking with assurance that it will be meaningful

Many etiological agents in cigarette smoke have been proposed polynuclear hydrocarbons, arsenic, nitrosamines, and polonium Although fashion has turned somewhat against polynuclear hydrocarbons, it remains a strong contender, especially with the abundance in cigarette smoke of candidates for potent promoting factors This is not to say that there may not be other initiating carcinogens, such as nitrosamines, contributing to the overall outcome

A reliable determination of the etiological agents for lung cancer in cigarette smoking remains one of the most important public health issues and scientific challenges of this time Accordingly, these studies urgently need continued pursuit

The epidemiological evidence linking community air pollution to lung cancer remains ambiguous In the first instance, it is clear that the contribution from air pollution, if any, is very much less than that resulting from cigarette smoking Virtually all studies, however, that have been adequately conducted so that comparison is limited to groups comparable in cigarette smoking show that urban dwellers have a somewhat higher lung cancer rate than those from rural areas Thus, in one study,[10] representative results for standardized mortality ratios for lung cancer in males are 8 for nonsmokers from rural areas compared to 25 for urban nonsmokers, for smokers consuming more than one pack a day the rural ratio is 367 compared to the urban ratio of 621 These have been normalized by taking the lung cancer ratio for white males over 35 as equal to the reference value of 100 This urban difference, of course, could be due to factors other than air pollution The fact that the relatively small urban increase in lung cancer cannot be quantitatively related to the extent of atmospheric pollution among cities[11] teaches us little more than that attempts to correlate small signals extracted from a large background of noise are likely to lead to indeterminate outcomes

At this time, I think we must assume that the case is unproven, but we must accept the possibility that a modest air pollution contribution to lung cancer may exist

Experimentally, we do know that community air contaminants contain polynuclear hydrocarbon carcinogens Kotin and Wisely[12] have produced lung

cancer with ozonized gasoline superimposed on influenza infection in mice. Our own laboratory, as will be discussed elsewhere in this program, has shown that polynuclear hydrocarbons plus sulfur dioxide jointly produce cancer in rats at concentrations which do not produce such an outcome when either is administered alone.[9] The levels used were much higher than those found in even heavily contaminated cities. On the other hand, of course, the incidence rates observed in the laboratory study were vastly higher than those possibly attributable to air contaminants by present epidemiological data. Perhaps the most important implication of these studies is the mechanism whereby a simple irritant such as SO_2 can, at fairly low levels, convert a negative into a positive outcome. Such irritants may be very important co-acting factors.

If one thinks of the urban difference in lung cancer as (1) stemming from community air pollution and (2) as primarily attributable to polynuclear hydrocarbons, we should not be surprised at the relatively low incidence levels which can be maximally attributed to it if examined in the light of the experience of those occupationally exposed in gas works to such compounds at much higher levels. Careful epidemiological studies of individuals fairly heavily exposed to polynuclear hydrocarbons in gas production plants have shown a real but relatively modest increase in incidence. The most systematic studies suggest that when the most heavily exposed individuals are compared with those having no such exposure, there is about a 70% increase in incidence.[13] An increase of this magnitude in a disease having the high prevalence of lung cancer is relatively hard to measure.

Experimentally the polynuclear hydrocarbons that we have been speaking of are unequivocably capable of producing lung cancer in animals by implant, yielding an orderly dose response curve with pellet implants of the polynuclear hydrocarbons at various dilutions in the carrier cholesterol.[9] As alluded to above, inhalation of polynuclear hydrocarbons by itself has not experimentally produced lung cancer, but its joint inhalation with an irritant has done so. An ingenious technique using an iron oxide carrier for polynuclear hydrocarbon yields lung cancer in the hamster.[14]

Occupational exposure to mustard gas[15] has been shown to be associated with an increased incidence of lung cancer. Compounds of this type are capable of enhancing the incidence of adenomata in mice.[16] I am not aware of any experimental production of bronchogenic cancer with such compounds.

Evidence in Germany many decades ago of lung cancer from chromium manufacture was largely disregarded in this country until the studies of Machle and Gregorius in 1947,[17] which demonstrated an increase in lung cancer incidence in chromium chemical manufacture. This was confirmed in English plants a few years later.[18] In this occupational situation, the most urgent need has been specification of the agent or agents responsible for the disease so that reliable protection during manufacture could be designed. Experimental work has been mixed, ranging from negative results even with adenoma-prone mice[19] to low yields of tumors from implants in the pleural cavity.[20] More recently,

work in our own laboratory, using the promising but still not widely used technique of bronchial pellet implantation devised by Kuschner and Laskin, has indicated a positive outcome, also in low yield, with calcium chromate (6/100), possibly process residue (1/100) and, curiously, hepatocell carcinoma (2/100) with chromium trioxide.[9] This work suggests that there may be active agents at several stages in the process and, therefore, the manufacturing process cannot be simply controlled but may require protection at a number of points.

Epidemiological evidence has suggested that lung cancer may arise from exposure to arsenic in a variety of situations – earlier, in the manufacture of arsenic pesticides;[21] later, in vineyard workers;[22] and more recently, in those occupationally exposed as smelter workers.[23] In the latter, excess cancer rate was as high as eightfold in employees who were under exposure 15 years or longer. Exposure to arsenic in this study was associated with exposure to sulfur dioxide and to other materials as well. Lee and Fraumeni[23] draw attention to the experimental work in our laboratory (noted above) showing cancer production with joint exposure to benzpyrene and SO_2.[9] There is no experimental evidence demonstrating the carcinogenicity of arsenic compounds for the lung.

Workers in nickel refineries have been found to have a high incidence of lung and nasal cancer.[24] In this instance, the work of Sunderman[25] showed that nickel carbonyl produces bronchogenic cancer in rats exposed by inhalation.

Beryllium compounds, especially the sulphate, have now been shown capable of producing lung cancer experimentally in rodents[26] and monkeys (see ref. 26). Although there is some inconclusive epidemiological data, evidence that this constitutes a lung cancer hazard to beryllium workers has not been forthcoming.

Evidence that asbestos exposure can lead to an increased incidence of lung cancer has been present for a long time. More recently, mesothelioma, a normally very rare lesion, has been demonstrated in association with asbestos exposure (see Cooper[27] for review). Both lesions have now been produced in experimental animals, the mesothelioma by Wagner[28] and bronchogenic cancer by Gross *et al.*[29] As in the case of uranium miners noted above, a strong synergistic role of cigarette smoking has been shown.[30]

Finally, three other situations should be listed in which cancer of the respiratory tract has been reported in man. These were associated with hematite mining,[31,32] the manufacture of isopropyl alcohol,[33] and wood-working.[34]

This quick scanning of human experience and the associated experimental work has not been presented as definitive but has been intended to illustrate the accomplishments which, though modest, have yet been substantial in our understanding of lung cancer from environmental sources and, at the same time, the considerable distance we must travel to achieve the assurance required for the control of this group of important health hazards.

In a current assessment, it would seem clear that although we are now in a position to come to grips experimentally with a number of the intricate controlling factors which determine the outcome in experimental animals, we

are far from having adequate tools for reliable evaluation. We can now with more confidence ask rather narrowly defined questions about potential lung cancer hazards. However, the problems of developing an experimental model is far from being simple and straightforward. This is true not only for dosimetric factors but also relates perhaps especially to promoting factors which may be of very substantial, and sometimes overriding, importance. For all of these reasons, I think it quite unlikely that we will ever be able to have a single "laboratory model" for lung cancer, in the sense that one has a box with animals into which we put our agent and from which we withdraw our animals at suitable times for examination. The dissimilarities in anatomy, tissue responsivity, etc., between the experimental animal and man are in most instances too great to permit such a highly desirable but elusive simplification.

Of continuing urgency for still some time will be careful study of the experimental models which are now evolving and others not now tried, so that we can have a fuller grasp of their capabilities and their limitations. This will involve an understanding of the natural history of development of the experimental lesions, the biological responsivities of the target tissues, and the anatomic and functional characteristics of the lung, all of which play decisive roles in controlling effective doses to the affected tissues, the reference species throughout being man.

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REFERENCES

1. E. Lorenz, Radioactivity in lung cancer: A critical review of lung cancer in the miners of Schneeberg and Joachimsthal. *J. Natl. Cancer Inst.*, 5 (1944) 1–15.
2. A Pirchan and H. Sikl, Cancer of the lung in miners in Jachymov (Joachimsthal). *Am. J. Cancer*, 16 (1932) 681–722.
3. B. Altshuler, N. Nelson, and M. Kuschner, Estimation of lung tissue dose from the inhalation of radon and daughters. *Health Phys.*, 10 (1964) 1137–1161.
4. S. Laskin, M. Kuschner, N. Nelson, B. Altschuler, J. H. Harley, and M. Daniels, Carcinoma of the lung in rats exposed to the beta radiation of intrabronchial ruthenium-106 pellets. I. dose-response relationships. *J. Natl. Cancer Inst.*, 31 (1963) 219–231.
5. F. J. Burns, R. E. Albert, and R. D. Heimbach, The RBE for skin tumors and hair follicle damage in the rat following irradiation with alpha particles and electrons. *Radiation Res.*, 36 (1968) 225–241.
6. Federal Radiation Council, Radiation Exposure of Uranium Miners, Report of an advisory committee of the National Academy of Sciences National Research Council, General Services Administration. Washington, D.C., 1968.

7 W J Clarke, J F Parke, J L Palotay, and W J Bair Plutonium inhalation studies VII. Bronchiolo alveolar carcinomas of the canine lung following plutonium particle inhalation *Health Phys* 12 (1966) 609 613

8 C L Yuile, H L Berke and T Hull, Lung cancer following polonium 210 inhalation in rats *Radiation Res* 31 (1967) 760–774

9 M Kuschner, The J Burns Amberson lecture The causes of lung cancer *Am Rev Respirat Diseases*, 98 (1968) 573–590

10 W Haenszel D B Loveland, and M G Sirken, Lung cancer mortality as related to residence and smoking histories I White males *J Natl Cancer Inst* 28 (1962) 947 1001

11 P Buell, J Dunn, and L Breslow, Cancer of the lung and Los Angeles-type air pollution *Cancer* 20 (1967) 2139–2147

12 P Kotin and D V Wiseley, Production of lung cancer in mice by inhalation exposure to influenza virus and aerosols of hydrocarbons In, *Progress in Experimental Tumor Research* Vol 3, pp 186 215, F Homburger (ed) S Karger, Basel/New York, 1963

13 R Doll, R E Fisher, and E J Gammon, Mortality of gasworkers with special reference to cancers of the lung and bladder, chronic bronchitis and pneumonoconiosis *Brit J Ind Med* 22 (1965) 1 12

14 U Saffiotti, Experimental respiratory tract carcinogenesis In, *Progress in Experimental Tumor Research*, Vol 11, pp 302–333, F Homburger (ed) S Karger, Basal/New York, 1969

15 S Wada, Y Nishimoto, M Miyanishi, S Kambe, and R W Miller, Mustard gas as a cause of respiratory neoplasia in man *Lancet* 1 (1968) 1161 1163

16 W E Heston, Pulmonary tumors in strain A mice exposed to mustard gas *Proc Soc Exptl Biol Med*, 82 (1953) 457–460

17 W Machle and F Gregorius, Cancer of respiratory system in United States chromate producing industry *Public Health Rep*, 63 (1948) 1114–1127

18 P L Bidstrup and R A M Case, Carcinoma of the lung in workmen in bichromates-producing industry in Great Britain *Brit J Ind Med* 13 (1956) 260 264

19 C H Steffee and A M Baetjer, Histopathologic effect of chromate chemicals *Arch Environ Med* 11 (1965) 66–75

20 W G Hoeper and W W Payne, Experimental studies in metal carcinogenesis Chromium, nickel, iron, arsenic *Arch Environ Health*, 5 (1962) 445–462

21 A B Hill and E L Faning, Studies in the incidence of cancer in a factory handling inorganic compounds of arsenic *Brit J Ind Med*, 5 (1948) 1–15

22 F Roth, The sequelae of chronic arsenic poisoning in Moselle Vintners *German Med Monthly* 2 (1957) 172–175

23 A M Lee and J F Fraumeni, Jr, Arsenic and respiratory cancer in man an occupational study *J Natl Cancer Inst*, 42 (1969) 1045–1952

24 Richard Doll, Cancer of the lung and nose in nickel workers *Brit J Ind Med* 15 (1958) 217

25 F W Sunderman and A J Donnelly, Studies of nickel carcinogenesis metastasizing pulmonary tumors in rats induced by the inhalation of nickel carbonyl *Am J Pathol* 46 (1965) 1027–1041

26 A L Reeves, D Deitch, and A J Vorwald, Beryllium carcinogenesis I Inhalation exposure of rats to beryllium sulfate aerosol *Cancer Res* 27 (1967) 439–445

27 W C Cooper, Asbestos as a hazard to health *Arch Environ Health*, 15 (1967) 285–290

28 J C Wagner, Experimental production of mesothelial tumors of the pleura by implantation of dusts in laboratory animals *Nature*, 196 (1962) 180–181

29 P Gross, R T P de Treville, E B Tolker, M Kaschak, and M A Babyak, Experimental asbestosis *Arch Environ Health* 15 (1967) 343–355
30 I J Selikoff and others, Mortality experiences of asbestos insulation workers In, *International Conference on Pneumoconiosis Johannesburg* pp 97–103, 1969
31 J S Faulds, Hematite pneumoconiosis in Cumberland miners *J Clin Pathol* 10 (1957) 187
32 M L Levin, A S Kraus, I D Goldberg, and P R Gerhardt, Problems in study of occupation and smoking in relation to lung cancer *Cancer* 8 (1955) 932–936
33 C S Weil, H F Smyth, Jr , and T W Nale, Quest for suspected industrial carcinogen, *A M A Arch Ind Hyg Occupational Med* 5 (1952) 535–547
34 E D Acheson, E H Hadfield, and R G Macbeth, Carcinoma of the nasal cavity and accessory sinuses in woodworkers *Lancet* I (1967) 311–312

CHEMICAL DEFINITIONS OF INHALATION HAZARDS

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ABSTRACT

The chemical composition and physical characteristics of aerosols, dusts, and gases may be of comparable importance as factors leading to diseases of the lung Environmental pollutants often work together in synergistic fashion to alter cells and tissues towards the development of various disease states that may prove irreversible —— Individual compounds will be considered for their role in reducing effective defense mechanisms of the lung and for their contribution towards permanent changes that ultimately lead to emphysema or cancer This review will also deal with factors which were originally associated only with specific occupations but which have tended to become generalized hazards for the urban population, such as exposure to asbestos fibers, beryllium, nickel, etc It will deal with involuntary as well as voluntary air pollution – auto traffic pollution and cigarette smoking – estimating the importance of each and identifying where possible the role of significant components in the development of disease The contribution of various dusts, even "inert carriers," as well as that of simple organic chemicals of ubiquitous occurrence in polluted air and that of carcinogens responsible for irreversible changes in susceptible cells, will be discussed — Pollutants in the air we breathe are considered for their enzyme inducing capacity as well as for their inhibitory action Physiological changes leading to tolerance are explored as not necessarily being free of hazards Viral infection and air pollution have reciprocal action that can contribute toward disease of the lung

The topic "Chemical Definitions of Inhalation Hazards" is like a jigsaw puzzle that is worked on, piece by piece, by somebody with time to spare and patience to try who realizes slowly that some of the puzzle pieces are cut on a different scale, are in different dimensions, are flexible and pliable, and that the whole puzzle is four-dimensional Like such a puzzle, the problem before us is extremely hard to tackle

The factors that lead to cancer of the lung in man are multiple I do not want, however, to focus immediately on interactions such as synergisms, which

remain ill-defined in the context of lung cancer induction, but will, instead, discuss other much simpler mechanisms that are overcome before the carcinogen could reach the target cell, before the target cell would be responsive, and before with time the environment would provide the triggering events to lead to overt cancer. In this presentation the focus is often on problems that may appear to the listener to be far removed from pulmonary carcinogenesis, the real topic for discussion at this symposium. However, I hope that this presentation will convey to you the reason for our concern with these problems, and why they appear to be of such importance.

When it became evident that most of the carcinogenic agents do not readily produce lung cancer in animals when inhaled, the first explanation that could be offered was that the defense mechanisms of the respiratory system in all experimental species were very adequate for the protection of the lung by mechanisms that had been explored, and were partly understood. We should, therefore, focus some attention on those chemicals that may alter and thus impair these processes. To begin, we must be aware of the physical state of the toxic agent before we are concerned with its chemical properties and resulting biological toxicity.

Toxic chemicals that can affect the tracheobronchial tree are inhaled on many occasions. There are those that we deliberately want to inhale, such as cigarette smoke, those that we do not want to inhale, but inhale because they are around us – such as cigarette smoke – and those such as urban air pollution that we can hardly avoid inhaling if we want to get around in congested traffic.

It has been brought to light that the mucous sheath in the tracheobronchial tree can be adversely affected by pollutants. This may be the first breech of the defenses (*i.e.*, the normal continuous movement of the mucus in a cephalad direction to remove from the tracheobronchial tree all foreign impacted particulates) which has been well documented.

Chemicals that adversely affect the mucus are simple acids that will actually precipitate mucoproteins and prevent proper movement of the mucus. But other chemicals also affect mucous flow. Most of these, however, affect the rate of secretion of mucus and cause changes in its consistency or ultimately affect ciliary activity.

It has not always been realized that ciliary activity of the tracheobronchial tree is quite capable of remaining unaltered throughout many of the toxic insults. Ciliary activity can, of course, be stopped by hydrogen cyanide and other environmental chemicals, but mucous flow will have stopped long before the cilia are defeated. Mucostasis, rather than ciliostasis, is therefore one of the first trouble spots in the pulmonary defense system.

The possibility that synergism between SO_2 and carbon particles produces enhanced inhibition of ciliary activity was studied by Dalhamn.[1] He found that the inhibition was entirely due to SO_2. There was no synergism. The idea of synergism between particulates, aerosol, and gases, in their toxic action on the

tracheobronchial tree, nonetheless persisted and has been further explored, but there is no suggestion that such interactions affect the ciliary function adversely.

Boren[2] has described very significant interactions between NO_2 and carbon particles in the production of focal destructive pulmonary lesions in Swiss albino mice. They were localized and severe, and were not observed upon inhalation of NO_2 alone. Here, then, is an example of the particulates contributing considerably to the toxicity of an irritant. Carbon particles and NO_2 were not considered to be unique for that synergistic interaction.

One needs to realize that the synergistic effects of aerosols, particulates, and gases have to be studied for every factor at realistic concentration levels as they may be encountered, because extrapolation to ambient air conditions is often too formidable a task. In reviewing the literature, however, one is struck by the fact that experiments have usually been designed with high dose levels, and results often have been extrapolated freely towards realistic situations. Extrapolation to man often brings additional difficulties.

Air pollutants that will affect pulmonary flow resistance were studied by Frank *et al.*,[3] who showed that it makes a difference whether the experiment is done on guinea pigs or on man. Human volunteers experienced no enhanced ill effects from inhalation of 1–17 ppm SO_2 and NaCl aerosols, compared to SO_2 alone. However, inhalation of sulfur dioxide and a NaCl aerosol in the guinea pig augmented toxicity considerably.[4] One realizes that the susceptibility of different species to such comparatively simple combinations of toxic agents was not readily predictable, and therefore cannot be extrapolated from one species to the next. The guinea pig seems specifically susceptible to bronchial spasms after inhaling sulfuric acid mist, and the enhanced toxicity was caused by the oxidation to H_2SO_4 of part of the SO_2, when in contact with the sodium chloride aerosol.[5] This example is mentioned as one of the pieces of the complicated overall picture in an effort to focus on the difficulties in evaluating such simple problems as: What is the synergistic countribution of the particulate matter in the air towards the toxicity of SO_2, at such low levels as are normally encountered (*i.e.*, about 0.2 ppm SO_2).

Another interaction of carbon particles with air pollutants deserves mentioning. High concentrations of soot (350 mg/m^3), produced by smoky kerosene lamps, and then SO_2 (1000 ppm) were inhaled by mice. The kerosene smoke protected against the toxicity of SO_2 when a 23-hr time interval was interspaced. If the two pollutants were given together, there was no protection, but rather increased toxicity in an additive instead of synergistic fashion.[6] These observations suggested to other investigators that one should study this phenomenon using more definitive substances.

Wagner *et al.*[7] gave animals mineral and motor oil mists as inhalants before exposing them to ozone or NO_2 and observed protection against these toxic air pollutants. In studies of this type it is important to know the dose levels, the duration of protection, the species specificity, and other parameters. The

particle size of the oil mists, which ranged in diameter from 0.5 to 1 μ was of major importance for effective protection. Particles of this size would pass through the nares into the lung. The oil mists were introduced at high dose levels (72–90 mg/m^3) and actually protected the mouse against exposure to 6 ppm O_3, or 90 ppm NO_2 given 18 hr later. The protection lasted 8 days, becoming weaker with time. Inhalation of mineral oil will not protect against the toxicity of ozone or NO_2 if the interval between oil mist inhalation and oxidant is less than 8 hr.

In these mineral oil pretreatment experiments, the ozone challenge still produced tolerance, suggesting that some of the ozone penetrates to the responding tissue, so that the lack of toxicity does not imply complete reaction with or adsorption by the mineral oil. There is no explanation available for the protection mechanism, except for the suggestive finding that the amount of mineral oil inhaled is adequate for a complete monomolecular coverage of the alveolar surfaces.

LaBelle *et al.*[8] studied formaldehyde, acrolein, and nitric acid as typical air pollutants, and evaluated different aerosols for their modification of the toxicity of these gases to mice. He attempted to predict increases or decreases in toxicity due to the gas and aerosol particle combination, on the basis of the difference in depth of penetration between the aerosol particles and the gases. His theory was only 60 to 70% capable of predicting the results correctly. This experiment gave information on how far toxic vapors would penetrate into the lung, to what extent they would be adsorbed on aerosol particles of certain sizes, and to what extent this would change their toxicity upon inhalation. Adsorption on aerosols increased the toxicity of formaldehyde, but decreased that of nitric acid.

LaBelle and Brieger[9] also studied factors in the clearance of various dusts from the lung. They were interested in evaluating particle size variation, chemical composition, and the overall effectiveness of phagocytosis. The smaller the particles, the longer was their retention time in the lung, and the greater was the stimulation of phagocytosis. This generalization, however, is not necessarily true.

Work reported recently by Bingham[10] showed that the number of pulmonary macrophages was severely depressed in 24 hr when rats inhaled an aerosol of lead sesquioxide at 150 μg/m^3. Lead oxide was also tested at 10 μg/m^3 to allow inhalation of lead at dose levels encountered in ambient city air. The depression of pulmonary macrophages reached the same low level only after 8 days. These workers noted that the macrophages which were found were functional, but that this low level (*i.e.*, 20% of normal) remained constant as long as the animals inhaled lead sesquioxide at a concentration of 150 μg/m^3. When the animals were returned to clean air, the pulmonary macrophages returned to normal levels in 3 days. This observation has so far not been extended to other chemicals that are present in our atmosphere. The earlier studies by LaBelle never explored chemical properties in depth, but focused mainly on the significance of particle size.

Protection against acute toxicity of air pollutants has been determined experimentally and has been interpreted in a positive and optimistic fashion. One might assume that air pollution of the Los Angeles type may not be as toxic to the local inhabitants as to occasional visitors, since frequent exposures to ozone or to NO_2 produce tolerance to ever increasing doses of oxidants at subsequent encounters of air pollution. Apparently animals will not only survive 10 times the dose of oxidant that would have killed them if it had been their first exposure, but can also be protected over a period of time These oxidants also turned out to be cross-protective One air pollutant could initiate protection against toxicity due to other air pollutants Cross-protection has also been established for ketene, ozone, and NO_2 [11-13] Each protected the animals well against lethal doses of the others. Such protection has also been seen in man.[14]

The explanations offered in the literature are not very convincing, but the theories underlying protection are well discussed in a paper by Fairchild.[15] The main reason for presenting this topic of protective action against acute toxicity of air pollutants is the premonition that this protection against the hemorrhage- and edema-producing effects of ozone or NO_2 will still leave much of the chemical in contact with tissue The modified response of the target tissue may harbor new problems due to this adaptation which in the long run may even be more hazardous than the acute toxicity would have been

Another interesting experiment involving "protection" has been carried out by Ottolenghi and Thompson,[16] who observed protection in rats which had been injected intravenously with a high dose of piperonyl butoxide, a pesticide synergist (2.5 ml/kg), 15 hr before inhalation of O_3 at a 1 ppm dose level for 4 hr The pulmonary response to O_3 would have killed the animals, by producing pulmonary edema, but the piperonyl butoxide exposure protected them. However, the combined exposure produced changes in the lung which were reminiscent of hyaline membrane disease. These findings persisted throughout the observation period of 3 weeks, but since these were short-term experiments, one does not know the outcome of the long-range chronic exposures to this combination of a pesticide synergist and an oxidant. This kind of change in response and reaction makes one wonder again whether tolerance may not have its bad side too.

One must also pay attention to the effects of these air pollutants on viral and bacterial infection of the respiratory tract in relation to man. Since this problem will be discussed by subsequent speakers, it will only be touched upon lightly. That man has been affected adversely by air pollution has been observed from his epidemic bouts with influenza in England[17] at various times in the 1950's, by heightened mortality in the U.S., particularly in New York,[18] and by the incidence of respiratory illness in Pittsburgh.[19] A positive correlation between air pollution and human respiratory infections refers specifically to lower respiratory tract infections in a study on several thousand school children in England [20]

Laboratory studies carried out by Coffin[21] with O_3, and by Ehrlich[22] and others[23] with NO_2, have established that nearly realistic concentrations of oxidants have serious effects on the survival of mice, hamsters, or monkeys subsequently exposed to aerosols of various bacterial organisms. The lowered resistance due to, for instance, a 3-hr exposure to 4 ppm of ozone inhaled for several hours would last for a period of up to 18 hr thereafter, leading to increased susceptibility of the animal to the bacterial organism.[24]

Artificial smog made by allowing O_3 to react with vaporized gasoline was inhaled by C57BL mice that were also given successive infections with three mouse-adapted strains of influenza virus. The progression of changes in the lung, from the initial proliferative response to the virus infections through squamous metaplasia with keratinization to squamous cancer, were described. Only in the group of mice exposed to both influenza viruses and smog were epidermoid carcinomas observed.[25]

The effect of myxovirus infection in mice as potentially modifying factors on the effect of air pollution or inhalation of asbestos will be taken up by other speakers at this conference.

Apart from the effects of air pollutants on microorganisms, another interesting problem relates to the effect that pesticides may have on viruses. The studies[26] reported here, however, were carried out without relevance to the lung. Human Chang-strain liver cells in tissue culture were infected with vaccinia and polio virus. At subtoxic concentrations DDT, chlordane, malathion, dicofol, and dinocap inhibited replication of vaccinia. At these same low concentrations dicofol and dinocap stimulated replication of polio virus, while the other agents proved to be inhibitory. This sort of information, although not directly related to our quest, is very disconcerting in its implication.

The composition of tobacco smoke with regard to chemicals that have a carcinogenic effect will be discussed by others. The gas-phase components of cigarette smoke are also of considerable importance. They are listed in Table 1 in order of probable importance, based on concentration and toxicity, so that a compound present in smoke at comparatively low concentration, but high toxicity, may rank at the same level as one present in very high concentration, but low toxicity. The first on the list is carbon monoxide. It is present in high concentration in cigarette smoke and has multiple toxic effects, so that it represents the compound par excellence that should be preferentially removed if techniques could be developed. Carbon monoxide has been found to be a capable inhibitor of microsomal enzyme induction, blocking the P-450 factor,[27] and thus a very important system in protecting the lung against various toxic agents may be inactivated.

The next most important component I would say is NO, which has produced controversy for the simple reason that it is actually not extremely toxic, but it would be mentioned as a precursor of one of the most toxic compounds, since on leaving the cigarette, NO is partially oxidized to NO_2 to at least 30% in 1 min.[28] One must consider this an NO/NO_2 problem. NO_2 has chemical affinity

TABLE 1

Composition of Gas Phase of Cigarette Smoke and Estimation of the Importance of Removal of Its Compounds

Chemical	Average concentration in μg in smoke of one cigarette	Toxicity	Importance
Carbon monoxide	16,000	+3	+++
NO	400	+2	+
NO_2	0–200	+4	+++
HCN	300	+4	+++
Formaldehyde	40	+4	++
Acetaldehyde	1000	+2	++
Propionaldehyde	40	+1	–
Acrolein	45	+4	++
Methacrolein	8	+2	+
Crotonaldehyde	15	+3	+
Butadione	50	+2	+
1-Butenone-3	30	+3	+
1-Pentenone-3	45	+3	+
Formic acid	500	+3	++
Acetic acid	600	+2	+
Propionic acid	300	+1	–
Phenol	120	+1	–
Acetonitrile	140	+1	–
Propionitrile	30	+1	–
Acrylonitrile	10	+3	+
Methacrylonitrile	3	+3	–
Crontononitrile	3	+4	+
H_2S	10	+4	–
NH_3	100	+3	+

for constituents of most biological systems, and its toxicity is manifested by the ready destruction of membranes of cellular and subcellular structures. It may carry major responsibility for the high incidence of emphysema in cigarette smokers.

Hydrogen cyanide is a potent ciliary toxin. Its inhibitory action on respiratory enzymes is too well known for comment. Formaldehyde and acrolein also affect ciliary activity and mucous flow. These toxins are followed by a large number of minor components that are significant when considered together (*e.g.*, crotonaldehyde, butanedione, pentenone, acetonitrile, acrylonitrile, ammonia, etc.).

These gas-phase components of cigarette smoke are mentioned in detail because efforts to reduce the adverse health effects of cigarette smoking could be channeled in the direction of lowering these components without too much

delay and with some hope for success. Although removal of these components may have considerable impact on improving health, this step may be rejected by the smoker on the basis of undesirable changes in taste.

The toxicity of low-molecular-weight alkanes and alkenes that are also present in cigarette smoke and air pollution has been largely ignored. A combination of alkane and an alkene showed enhanced toxicity that was quite unexpected. Shugaev[29] studied the LD_{50} for mice and rats inhaling butane or isobutylene for 2 and 4 hr, respectively, and determined the concentration of each in the brain. When the two chemicals were inhaled together, the LD_{50} for both mice and rats was reached at much lower concentrations of the chemicals in the brain, suggesting a synergistic effect.

Early studies on polycyclic hydrocarbons, in which cancer was produced on the skin of mice, may have suggested that these chemicals would also produce cancer of the lung, provided the conditions were right. Results to the contrary have puzzled scientists and more will be heard about this problem later in the conference. Several speakers will elaborate on the conditions required for the carcinogenic effect to be observed.

Campbell[30] was among the first to use inhalation techniques to study the effects of carcinogenic materials in road dust on the respiratory tree and lungs in general. He exposed mice 4-6 times daily, 5 days a week, to a heavy cloud of dust obtained from the sweepings of tarred roads. The dust had first been sifted through a 100-mesh sieve. His mice developed both papillomas and epitheliomas, as well as lung tumors. In the first experiment, lung tumors developed in 26 out of 44 animals exposed to dust for 240 days. In a second experiment, they developed in 45 out of 56 animals exposed for 255 days, and in two control groups, they developed in 4 of 50 and in 3 of 40 mice.

In a subsequent experiment, Campbell[31] used inhalation of detarred road dust as his control. In these experiments lung tumors developed in 45 of 73 animals exposed to road dust and in 26 of 78 animals exposed to detarred road dust. Ten of 77 animals not exposed to dust developed lung tumors. The conclusion was reached that if the polycyclic hydrocarbons could produce lung tumors, the contribution of the "inert" dust was very important. Thus, there is a combination of factors at work, as Dr. Saffiotti will soon discuss [see these *Proceedings*, p. 27].

Simmons[32] studied asphalt inhalation in mice to see if he could produce lung tumors in his animals. They had to inhale asphalt every day for 400 days. He obtained negative data for the lung, but in separate experiments papillomas and epitheliomas developed in animals whose backs had been painted with benzene solutions of the same asphalt.[33] These experiments proved the presence of carcinogens in the asphalt that were only effective when they made adequate contact.

Tye and Stemmer[34] exposed C3H mice to aerosols of coal tar and to aerosols of the neutral fractions of coal tars, as well as to aerosols to which the phenolic fraction had been returned. After 46 weeks of exposure, pulmonary

adenocarcinomas (12%) were found only in animals inhaling the phenol-containing aerosol, and squamous metaplasia was three times more frequent in these animals than in those exposed to the phenol-free tar aerosol. The control mice showed no pulmonary tumors at all. These results suggest that phenols can act as cocarcinogens with polycyclic hydrocarbons not only on the skin of mice, the preferred target for cocarcinogenesis studies in the past, but also in their lungs.

SO_2 was recently found to be a tumorigenic agent by itself. Peacock and Spence[35] exposed LX mice, a strain of mice very susceptible to lung tumor development, to 500 ppm of SO_2 for 5 min, 5 days a week, and observed a doubling of the lung tumor incident in a period of 300 days. In the female animal of that strain, lung tumors do not occur spontaneously, but these animals developed lung tumors when they were exposed to this high dose of SO_2. In a similar study done earlier, where the SO_2 exposure was to 4 to 8 ppm, no tumors were observed.[36]

Later in this conference [p. 342, these *Proceedings*], Professor Laskin will present data on the cocarcinogenic effect of SO_2 exposure and benzpyrene inhalation in rats and hamsters.

Asbestos fibers produce cancers in man, and in animals, but as could have been predicted, scientists are at a loss to explain this carcinogenic activity on a chemical or physical basis. Harington and Roe,[37] using crocidolite that was highly contaminated with oils containing benzpyrene (up to 24 μg/100 g fiber), removed benzpyrene and the oils by solvent extraction and noted that mesothelioma induction by these extracted fibers was reduced by 50%. This finding suggests that the contribution made by the polycyclic hydrocarbon, entering adsorbed on the asbestos fiber, could not account for all the carcinogenic activity of the asbestos.

Other contaminants of asbestos have been suspected of being carcinogens, and it was suggested[38] that in the processing of asbestos, contamination by nickel and chromium occurs. Chrysotile was richest in these contaminants – containing 5000 μg Ni, 100 μg Cr/g fiber, which could possibly explain its high carcinogenic activity.[39] The fact remains, however, that asbestos fibers act as carcinogens by themselves, even though the mode of action is unknown.

Of considerable significance also is Selikoff's[40] observation that cigarette smoking acts synergistically with asbestos fiber inhalation in causing bronchogenic cancer in man. Nonsmoking or cigar- and pipe-smoking asbestos workers did not have lung cancer in a study of 370 asbestos workers, but the cigarette-smoking workers had nearly 10 times the expected incidence compared to the cigarette smokers not exposed to asbestos.

Lung cancer hazards for man due to industrial exposure, which was dealt with in some detail by Dr. Nelson [pp. 3–12, these *Proceedings*], has generally not been considered in this discussion, but I believe Table 2 is very informative in demonstrating the urban air pollution by inorganic dusts containing metals suspect of carcinogenicity on high industrial exposure. As in the case of asbestos,

the specific industrial hazard may soon become a general community hazard and needs early attention.

TABLE 2

*Summary of Average Concentration of Specific Metals in the Particulate of Air Samples Taken in the U.S. in 1964 and 1965**

	Concentrations in ng/m^3 (arithmetic average)
Fe	1580
Pb	790
Zn	690
Ni	340
Mn	100
Cu	90
V	50
Ti	40
Sn	20
As	20
Cr	15
Mo	5
Cd	<2
Be	<0.5
Co	<0.5

*From "Air Quality Data from the National Air Sampling Networks and Contributing State and Local Networks, 1964–1965." U.S. Department of Health, Education, and Welfare, 1966.

REFERENCES

1. T. Dalhamn and L. Strandberg, Synergism between sulphur dioxide and carbon particles. Studies on adsorption and on ciliary movements in the rabbit trachea *in vivo*. *Intern. J. Air Water Pollut.*, 7 (1963) 517–529.
2. H. G. Boren, Carbon as a carrier mechanism for irritant gases. *Arch. Environ. Health,* 8 (1964) 119–132.
3. N. R. Frank, M. O. Amdur, and J. L. Whittenberger, A comparison of the acute effects of SO_2 administered alone or in combination with NaCl particles on the respiratory mechanics of healthy adults. *Intern. J. Air Water Pollut.*, 8 (1964) 125–133.
4. M. O. Amdur, The physiological response of guinea pigs to atmospheric pollutants. *Intern. J. Air Water Pollut.*, 1 (1959) 170–183.
5. M. O. Amdur, The effects of various aerosols on the response of guinea pigs to sulfur dioxide. *Arch. Environ. Health,* 16 (1968) 460–468.
6. R. E. Pattle and F. Burgess, Toxic effects of mixtures of sulphur dioxide and smoke with air. *J. Pathol. Bacteriol.*, 73 (1957) 411–419.

7 W D Wagner, O J Dobrogorski, and H E Stokinger, Antagonistic action of oil mists on air pollutants *Arch Environ Health,* 2 (1961) 47–58
8 C W LaBelle, J E Long, and E E Christofano, Synergistic effects of aerosols *A M A Arch Ind Health* 2 (1955) 297 304
9 C W LaBelle and H Brieger, Synergistic effects of aerosols II Effects on rate of clearance from the lung *A M A Arch Ind Health,* 20 (1959) 100–105
10 Eula Bingham, E A Pfitzer, W Barkley, and E P Radford, Alveolar macrophages Reduced number in rats after prolonged inhalation of lead sesquioxide *Science* 162 (1968) 1297–1299
11 R M Mendenhall and H E Stokinger, Tolerance and cross-tolerance development to atmospheric pollutants, ketene and ozone *J Appl Physiol* 14 (1959) 923–926
12 M Kleinfield and C P Giel, Clinical manifestation of ozone poisoning, report of new source of exposure *Am J Med Sci* 231 (1956) 638–643
13 P J R Challen, D E Hickish, and J Bedford, Investigation of some health hazards in inert gas, tungsten arc welding shop *Brit J Ind Med* 15 (1958) 276–282
14 D Henschler, A Stier, H Beck, and W Neumann, Geruchsschwellen einiger wichtiger Reizgase (Schwefeldioxyd, Ozon, Stickstoffdioxyd) und Erscheinungen bei der Einwirkung geringer Konzentrationen auf den Menschen *Arch Gewerbepathol Gewerbe hyg* 17 (1960) 547–570
15 E J Fairchild, Tolerance mechanisms *Arch Environ Health* 14 (1967) 111–126
16 A Ottolenghi and G Thompson, personal communication
17 W H Bradley, W P D Logan, and A E Martin, The London fog of December 2–5, 1957 *Month Bull Minist Health,* 17 (1958) 156–166
18 L Greenburg, F Field, C L Erhardt, M Glasser, and J I Reed, Air pollution, influenza, and mortality in New York City *Arch Environ Health,* 15 (1967) 430–438
19 F C Dohan, The complexity of the relationship between air pollution and respiratory health In, *Proceedings of the National Conference on Air Pollution, Washington, D C December 1962,* pp 137–139
20 J W B Douglas and R F Waller, Air pollution and respiratory infection in children *Brit J Prevent Social Med ,* 20 (1966) 1–8
21 D L Coffin and E J Blommer, The influence of cold on mortality from streptococci following ozone exposure *J Air Pollut Control Assoc* 15 (1965) 523–524
22 R Ehrlich, Effect of nitrogen dioxide on resistance to respiratory infection *Bacteriol Rev ,* 30 (1966) 604–614
23 M R Purvis and R Ehrlich, Effect of atmospheric pollutants on susceptibility to respiratory infection II Effect of nitrogen dioxide *J Infect Diseases* 113 (1963) 72
24 M R. Purvis, S Miller, and R Ehrlich, Effect of atmospheric pollutants on susceptibility to respiratory infection I Effect of ozone *J Infect Diseases,* 109 (1961) 238–242
25 P Kotin, Production of lung cancer in mice by inhalation exposure to influenza virus and aerosols of hydrocarbons In, *Progress in Experimental Tumor Research,* Vol 3, pp 186–215, F Homburger (ed) S Karger, Basel/New York, 1963
26 J Gabliks, Insecticidal compounds Effects on replication of vaccinia and polio viruses in human Chang-strain liver cells *Arch Environ Health,* 14 (1967) 698–702
27. R W Estabrook, D Y Cooper, and O Rosenthal, The light reversible carbon monoxide inhibition of the steroid C21 hydroxylase system of the adrenal cortex *Biochem. Z ,* 338 (1963) 741–755
28 H Barkemeyer and F Seehofer, Zur Untersuchung der Gas-Dampf-Phase des Cigarettenrauches 2 Mitteilung Zur Bestimmung des Stickstoffmonoxids (NO) aus der Gasphase des Cigarettenrauches *Beitr Tabakforsch ,* 4 (1968) 278–282

29 B B Shugaev, The combined effect of aliphatic hydrocarbons, using butane and isobutylene as an example, with respect to the effective concentration in the brain tissue *Farmakol Toksikol (Moscow)* 30 (1967) 102 105
30 J A Campbell, Cancer of skin and increase in incidence of primary tumors of lungs in mice exposed to dust obtained from tarred roads *Brit J Exptl Pathol* 15 (1934) 287–294
31 J A Campbell, The effects of road dust freed from tar products upon the incidence of primary lung-tumours of mice, *Brit J Exptl Pathol* 18 (1937) 215 223
32 M H Simmers, Petroleum asphalt inhalation by mice *Arch Environ Health,* 9 (1964) 727 734
33 M H Simmers, E Podolak, and R Kinosita, Carcinogenic effect of petroleum asphalt *Proc Soc Exptl Biol Med*, 101 (1959) 266–268
34 R Tye and K L Stemmer, Experimental carcinogenesis of the lung, II The influence of phenols in the production of carcinoma *J Natl Cancer Inst* 39 (1967) 175–186
35 P R Peacock and J B Spence, Incidence of lung tumors in LX mice exposed to (1) free radicals, (2) SO_2 *Brit J Cancer (London),* 21 (1967) 606–618
36 J Morik, T Kemeny, and P Kertai, A levego kéndioxidtartalmának hatása normál és szivizomkarositott patkanyokra krónikus kiserletben (Effects of the SO_2 content of the air on normal rats and rats with injured heart muscle, in long term experiments) *J Air Pollut Control Assoc Abstracts* 10 (1964) 6331
37 J S Harington and F J C Roe, Studies of carcinogenesis of asbestos fibers and their natural oils *Ann N Y Acad Sci*, 132 (1965) 439–450
38 L J Cralley, R G Kennan, and J R Lynch, Exposure to metals in the manufacture of asbestos textile products *J Am Ind Hyg Assoc*, 28 (1967) 452–461
39 J S Harington, Chemical studies of asbestos *Ann N Y Acad. Sci*, 132 (1965) 31–47
40 I J Selikoff, E C Hammond, and Jacob Churg, Asbestos exposure, smoking, and neoplasia, *J Am Med Assoc* 204 (1968) 106–112

DISCUSSION

Y. Alarie: When you talk about tolerance with ozone, I do not see it in the very same light that you do Ozone tolerance has been shown in mice in experiments performed with high concentrations, and with death of the animal used as the end point On the other hand, when experiments were done at low concentration, and pulmonary function tests were used as the criteria of response, we couldn't see development of tolerance

So it seems that if a tolerance develops, it's at a quite high concentration. We see it only when we look at deaths, a situation very different from the one in which we look at very subtle changes picked up by various other criteria I do not say that it does not exist, but I am questioning its importance.

H. Falk: I can't give you an answer to that because, as I said in the beginning, I have no explanation of what tolerance means, but you at least pointed out to me that it may not mean very much in the first place

G. C. Buell: The proponents of tolerance and I have had a running battle for years, as you may or may not know, and I don't buy it In fact, one of the leading exponents of this proposition in Germany, Henschler, says that it's due

to the formation of edema, which acts as a physical barrier, and there has been no satisfactory mechanism proposed. I agree with Dr. Alarie.

I noticed you used the words *tolerance* and *adaptation.* Do you use them synonymously?

Falk: Not necessarily. I used them separately. I do not feel that I am up here to defend a mechanism which I have no way of understanding, either in terms of its mechanism or its meaning, but which I felt, if it did exist, should not be considered a positive asset. So when two of the experts tell me it might not exist at all, under realistic conditions, then it takes away some of my worry. You see, I was worrying that we would all become more or less capable of living in an atmosphere of oxidant and pollutant that, because of this type of adaptation, would appear harmless without being so. That's why I brought up this whole topic.

R. Rylander: I would like to comment on hydrogen cyanide as being one of the important agents in environmental inhalation toxicity. It is true that HCN is very toxic to cell cultures, phagocytes in nutrient solutions, etc., but if one considers experimental *in vivo* conditions, with a realistic exposure level, the toxicity might be considerably lower.

We performed some experiments a couple of years ago in which we compared normal cigarettes with cigarettes to which extra hydrogen cyanide was added. When we compared the cigarettes in an experimental situation, where the cilia of a clam experimental model were studied, we observed an effect (*i.e.,* an increased toxicity) due to the hydrogen cyanide. In an *in vivo* system with cats, where the ciliary activity in the trachea was studied, we could not find any difference between the two types of cigarettes. This was probably due to the absorption of the hydrogen cyanide in the mouth or in the mucus before it actually reached the ciliated cells. In earlier experiments on humans, we demonstrated that about 60% of the water-soluble compounds in the smoke are absorbed in the mouth cavity within 2 sec. In summary, I think that, when extrapolating observations concerning the inhalation toxicity of individual substances, one has to judge very carefully the experimental situation where the original toxicity data were obtained.

Falk: Thank you. I agree entirely with your discussion.

D. L. Coffin: I would like to make another comment concerning tolerance. I think tolerance to ozone can mean many things to many people, depending on their point of view, so that there cannot be an opinion regarding tolerance in all the biological phenomena which are evoked by exposure to ozone, but there is tolerance produced in animals expressed by mortality when the doses are sufficient. The mechanism of this lethality is generally supposed to be pulmonary edema.

However, there is evidence that tolerance can be evoked on a cellular level. I believe that Donald Pace has shown that when cells in tissue culture are exposed

to ozone and are subsequently exposed to a larger dose, there is a difference in the lethal effects of the ozone on the cells. This suggests that there is some effect on a cellular level, probably through some metabolic mechanism conferred by ozone.

In our own work we have data which suggest that the tolerance is conferred. When mice are exposed to ozone in rather moderate concentrations of $^1/_{10}$ ppm and are subsequently re-exposed to 1 ppm, there is a reduction in the mortality brought about by the interaction of a subsequent exposure with bacteria. This suggests that tolerance does occur in this system. I also think that Stokinger has pointed out in some of his work that when animals are continuously exposed to ozone, in spite of what he would call tolerance, there are chronic effects which occur.

C. Kensler: I would like to comment on Dr. Rylander's remarks on cyanide and cigarette smoke. I don't think there is any question but what there is enough cyanide in cigarette smoke to produce effects on ciliary function and mucous transport activity. But how much it affects the respiratory tract in man is a question that is impossible to answer in any quantitative fashion at this time.

We know that there are tremendous differences in individual sensitivity to agents of this and other kinds, and that there is a tremendous variation in the dose as a result of different habits of smoking, so that some individuals will obviously receive a much larger dose of this and other water-soluble materials, depending on how they smoke.

With cyanide, as well as with acrolein and other agents, I think the prudent route would be to treat it as a potential hazard, which it certainly is, and I would agree with Dr. Falk that to remove it from cigarette smoke, which can be done with adequate charcoal filters, would probably be a good step forward.

EXPERIMENTAL RESPIRATORY TRACT CARCINOGENESIS AND ITS RELATION TO INHALATION EXPOSURES

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ABSTRACT

Inhalation exposures in experimental respiratory carcinogenesis studies can be directed toward two major objectives (1) definition of penetration, distribution, retention, and fate of inhaled materials, and of related host responses, (2) induction of tumors (particularly respiratory tract tumors) in experimental animals, including evaluation of inhalation bioassays for carcinogenic effects — Critical selection of experimental conditions must include both of the following factors (1) adequate inhalation system technology to ensure control of experimental conditions and effective exposure of biological targets (e g , sufficient dosage at pulmonary level), (2) adequate sensitivity of biological models to ensure measurable and meaningful end points (e g , biometrically acceptable level of susceptibility to tumor induction) — For studies aimed at relating experimental exposures to the identification and control of causative factors for lung cancer in man, it is important to select specifically susceptible animal models in which induction of carcinomas from the respiratory epithelium can be experimentally reproduced Such animal models have been developed, using physical and chemical agents applied directly into the respiratory tract by various techniques (e g , pellet implantation, intratracheal instillations) Inhalation exposures have been repeatedly successful with radionuclides, but so far only a few instances of respiratory carcinogenesis by inhaled chemicals have been experimentally well documented (nickel carbonyl, beryllium asbestos, and certain *N* nitroso compounds) — Systemically acting carcinogens that selectively affect the respiratory epithelium have been identified and in some cases shown to interact with topical exposures with remarkable potentiation of carcinogenic effects — Definition of host response conditions and multiple, interacting pathogenetic factors can be used to develop animal models capable of highly sensitive, selective carcinogenic responses to respiratory exposures even at relatively low dose levels, such as can be provided by inhalation techniques

PART I – EVALUATION OF THE PROBLEM

Two years ago, in reviewing the field of experimental respiratory tract carcinogenesis at the International Symposium on Carcinogenesis and Carci-

nogen Testing in Boston,[1] I quoted the figures for lung cancer deaths in the United States for 1967. The projected figures for that year were 300,000 total cancer deaths, of which 50,000 would be due to cancer of the lung and bronchi: that meant that 137 persons died of lung cancer every day, or one every 10 minutes. Now, looking at the figures projected for 1969, we find that in just 2 years, mortality for lung cancer in the United States has risen from 50,000 to almost 60,000 deaths, which means 165 deaths every day, or one every $8\frac{1}{2}$ minutes. In the United States, lung cancer still represents the highest single cause of cancer deaths.

To effectively prevent such a major death toll, we need extensive studies on the causative agents, the conditioning factors, and the pathogenic mechanisms responsible for this type of cancer. A new operational research program to attack the lung cancer problem has been launched by the National Cancer Institute during the current year, and is now actively under way.[2]

A. Purposes of Inhalation Studies

Inhalation has been the main mode of exposure in man to agents known to be causally associated with an increased incidence of respiratory cancer. Tobacco smoking is the single most important factor to be considered, because of the enormous extent of its use and the high degree of epidemiologic evidence. Other environmental and occupational inhalation factors have been identified as cancer hazards and were reviewed in the preceding papers of this conference.[3,4]

Various types of respiratory tract cancers in man are associated with inhalation exposures – not only lung cancer but also cancer of the larynx, and – for certain types of occupational exposures – cancer of the nasal cavities. Mesothelioma, a neoplasm of the pleura, is also caused by an inhalation exposure, namely, to asbestos.

The experimental study of carcinogenesis in the respiratory tract is aimed primarily at identifying the causative correlations between neoplasms of various types and sites in the respiratory tract, their causative agents and conditioning factors. The ultimate purpose of such studies is to prevent respiratory cancers by one of the following approaches: (a) minimizing the exposure to the causative agents; (b) inhibiting their biological activation; (c) enhancing the host resistance to their effects.

Respiratory tract carcinogenesis is still a young field of research, as compared with studies on carcinogenesis in the skin, subcutaneous tissues, mammary gland, hemopoietic tissue, or the liver. The data acquired in many carcinogenesis studies on other organs and tissues represent a useful background and a valuable basis for our work in respiratory carcinogenesis. However, a whole range of factors peculiar to its morphological, biochemical, and functional characteristics require special study.

In considering the approaches to the experimental study of the correlation, observed in man, between inhalation exposures and the induction of cancer, we

are confronted with two sets of problems, the scope of which could be defined as follows:

1. Study of inhalation exposure conditions and their effects.
   a. Effects other than cancer, although possibly related.
   b. Carcinogenic effects.
      b-1. On organs other than the respiratory tract.
      b-2. On the respiratory tract.
2. Study of biological models.
   a. For the role of effects other than cancer, particularly in their relation to cancer induction.
   b. For carcinogenic effects.
      b-1. On organs other than the respiratory tract.
      b-2. On the respiratory tract.

B. Study of Respiratory Tract Carcinogenesis

We will focus our interest now more specifically on the problem of respiratory tract carcinogenesis. The primary approach to this problem must be aimed at the development and definition of experimental models, to be used for the following studies: (a) bioassay of suspected respiratory carcinogens; (b) definition of the processes responsible for the development of carcinogenic responses in the respiratory tract; and (c) evaluation of corrective actions (i.e., procedures and developments aimed at reducing the carcinogenic effect, such as reduction of exposures, inhibitory effects on carcinogenic processes, or changes of the host response).

What experimental tools do we have to work toward these general goals? Several tools have been developed, and they can be outlined as follows:

1. Techniques for exposure.
   a. Techniques other than inhalation.
      a-1. By routes other than the respiratory (e.g., systemic, feeding, transplacental, or at-birth exposures resulting in a target effect on respiratory tissues).
      a-2. By the respiratory route (e.g., intratracheal instillation or pellet implantation).
   b. Inhalation techniques.
2. Biological models.
   a. Other than the whole animal (e.g., tissue culture or organ culture systems or techniques based on isolated tissues).
   b. Animal models, selected for adequate response to each of the techniques listed above.

C. Study of Inhalation Carcinogenesis

The use of inhalation exposures, therefore, appears to be only one of the many tools available for the attack on the problem of respiratory carcinogenesis (I think of experimental inhalation exposures as a sort of ultimate tool) Much of what will be discussed here will relate to our need to become more sophisticated in our use of inhalation techniques for animal carcinogenesis studies My view has been for quite some time that methods of exposure of the respiratory tract, other than by inhalation, can be essential in developing our understanding of the factors involved in respiratory tract carcinogenesis

Inhalation exposures can be of use in the study of respiratory carcinogenesis when directed towards two categories of objectives

1 Short-term studies
   a To define penetration, distribution, retention, and fate of inhaled materials, using a variety of techniques and models
   b To define the related host responses (e g , phagocytosis and cellular reaction to inhaled particles, cell kinetic studies on the populations of various cell types, biochemical reactions at different levels)
2 Long-term studies on tumor induction in experimental animals, particularly for respiratory tract tumors
   a To define carcinogenic processes specific to the respiratory system
   b To develop and perform carcinogenesis bioassays for inhaled materials

In the course of this conference a wide range of inhalation technologies and a variety of animal models will be discussed The essential exercise for us all lies in the critical selection of the experimental conditions necessary and sufficient for each specific purpose of study This being a young field of research, we are still all learning our way around

Reasonable or good animal models have been used in conjunction with poorly defined, or totally inadequate techniques of exposure (e g , some of the work performed in my laboratory, using intratracheal instillation techniques, was quite empirical in the evaluation of physico-chemical characteristics of the test materials, such as particle size On the other hand, good or excellent exposure technologies have been used in conjunction with insensitive and sometimes totally inadequate animal models (e g , studies conducted for excessively short periods of time, on insufficient numbers of animals, without the necessary controls, on animals that develop extensive respiratory infections, etc)

Yet, by trial and error, by the tenacious efforts of a few exiguous groups of investigators who worked hard in this field, such progress has been made in the last decade or so, and particularly in the last few years, that we can now begin to see a clearer pattern emerging out of the experimental results The analysis of

these results has taught us how to reproduce in animals the major categories of respiratory tumors observed in man, and how to link the induction of certain types of respiratory tumors to definite categories of exposures.

D. Morphology of Respiratory Tumors

The World Health Organization has recently compiled a histological classification and documentation of the main morphologic lung cancer types in man;[5] it includes the following categories:

I. *Epidermoid carcinomas* (characterized by keratinization or by intercellular bridges)

II. *Small cell anaplastic carcinomas* (with the following subtypes: 1. fusiform cell type; 2. polygonal cell types; 3. lymphocyte-like or "oat cell" types; 4. others)

III. *Adenocarcinomas* (1. bronchogenic; 2. bronchiolo-alveolar)

IV. *Large cell carcinomas* (1. solid tumors with mucin-like content; 2. solid tumors without mucin-like content; 3. giant cell carcinomas; 4. "clear" cell carcinomas)

V. *Combined epidermoid and adenocarcinomas*

VI. *Carcinoid tumors*

VII. *Bronchial gland tumors* (1. cylindromas; 2. mucoepidermoid tumors; 3. others)

VIII. *Papillary tumors of the surface epithelium* (1. epidermoid; 2. epidermoid with goblet cells; 3. others)

IX. *"Mixed" tumors and carcinosarcomas* (1. "mixed" tumors; 2. carcinosarcomas of embryonal types, 3. other carcinosarcomas)

X. *Sarcomas*

XI. *Unclassified*

XII. *Mesotheliomas* (1. localized; 2. diffuse)

XIII. *Melanomas*

The distribution of tumor types observed in man varies in different studies, but the most frequent types presently are the bronchogenic epidermoid carcinoma (accounting for 50–60% or more of the total number of respiratory tumors) and the small cell anaplastic carcinoma, oat-cell type (which accounts for up to 25–30% of the cases). The other anaplastic carcinomas and the adenocarcinomas are considerably less frequent.

A review of the main morphologic types of tumors induced in experimental animals indicates that comparable histologic patterns can be obtained in

experimental models by appropriate treatments.[6-8] By intratracheal administration of polynuclear hydrocarbons in rats and hamsters, a prevalence of bronchogenic squamouse cell carcinomas is obtained.[7,9-17] Certain systemic carcinogens, particularly diethylnitrosamine (DEN) in hamsters, give rise to adenomatous tumors of bronchial and bronchiolo-alveolar origin, as well as to papillary tumors in the trachea.[9,18-23] This effect appears to be dependent on a selective species specificity and so far it has only been noted in hamsters. Similarly, the well-known effect of urethan in inducing bronchiolo-alveolar adenomas in mice is not reproduced in rats or hamsters.

Of the main types of respiratory tumors seen in human pathology, only one, the oat-cell carcinoma, has not been found to be reproducible in experimental models. However, we have recently observed two cases of pulmonary small cell carcinomas in hamsters that were treated at birth with DEN;[19] two similar tumors developed in hamsters that had been exposed to the same carcinogen during their fetal life by the transplacental route, and were observed by Dr. Katherine M. Herrold.[24] The coincidence of these two preliminary findings suggests the possibility that the small cell carcinoma may derive from systemic carcinogenic stimuli acting during early periods of life.

In summary, we can now state that the major types of tumors of the respiratory tract, observed in man, can be reproduced in experimental animal systems.

E. *Selection of Experimental Systems*

The key to an effective experimental approach, aimed at identifying the causative factors responsible for the various types of respiratory tumors, is to be found in a selective and critical use of adequate exposure technologies and of adequate animal models susceptible to the induction of the desired tumor response.

In inhalation carcinogenesis studies, adequacy must be evaluated with consideration given to both these aspects, and their relation to each other. How should we evaluate their adequacy? I would like to offer some criteria for discussion.

1. Inhalation technology systems will be adequate for carcinogenesis studies if they ensure the following prerequisites:
   a. Definition, control, and reproducibility of experimental conditions (e.g., concentration, homogeneity, particle size, and other characteristics of the materials; effective monitoring methods, analytical methods, constant analytical results in time).
   b. Effective exposure at the level of the intended biological target (e.g., the eye for eye-irritation tests, the lung or bronchi for bronchopulmonary carcinogenesis tests).

b-1. Qualitative criteria, providing that the test material reaches the target tissue and that it does so in the intended form (e.g., an aerosol does not condense before reaching the target site; a chemical does not undergo oxidation or other unwanted reactions).

b-2. Quantitative criteria, providing that the administered material reaches the biological target in amounts possibly measurable and sufficient to elicit the intended effect (e.g., sufficient dosage of inhaled material at the pulmonary level).

2. Sensitivity of the biological models will be adequate if it ensures these prerequisites:
   a. Qualitative adequacy, i.e., the ability of the target tissue to develop the intended qualitative response (e.g., tumors of a certain type). This condition is provided for by the appropriate use of positive controls, which will demonstrate whether a certain tissue is capable of the intended type of response, possibly under even stronger conditions of exposure.
   b. Quantitative adequacy, i.e., the ability of the target tissue to develop such a response at quantitatively meaningful levels (e.g., number of animals sufficient to detect an expected incidence level; sufficient duration of the test and of the time of observation for an intended effect to have time to develop, as in the case of lifetime animal experiments for the detection of late-developing tumors).

Evaluation of carcinogenesis studies, especially those using relatively low exposures or exposures to relatively weak carcinogens, must be based on a determination of the validity of the design of these experiments in terms of proper biometric planning.

Many cases in the literature can be cited where a "negative result" is reported: a certain exposure technique has been set up, animals have been exposed and kept for a sufficient period of time, and no tumors have been produced. It is essential to recognize that this observation does not prove that the exposure was altogether ineffective. For example, if no tumors have been observed in two groups of 100 animals, one a negative control and one a treated group, this apparently "good" negative result only provides assurance, at the 99% probability level, that the true risk for tumors in the treated animals did not exceed 4.5%. If it had been 4% or less, it could very well not have been detected. Therefore, the value that we can attach to a so-called negative result in the literature is strictly dependent on the context of what has been done, and of how well it has been done.

In this connection we should discuss, as a general problem, the question to what extent one should try and develop sophisticated techniques to "mimic the environment" in the design of exposures for experimental carcinogenesis tests. Elaborate experiments have been designed at times to submit animals to conditions of exposure, such as diets or artificial atmospheres, that reproduce a

"perfect microenvironment." If, however, the animals chosen as targets are incapable of responding with a measurable biological effect, the enterprise becomes a meaningless exercise. Attempts to "mimic the environment" have been a very common occurrence in inhalation studies where a high refinement of methodologies has made it possible to reproduce in an inhalation chamber certain types of urban air pollution, or certain occupational exposure conditions. This accomplishment may require sometimes a considerable feat of technological ingenuity. Yet this technical refinement may become totally incongruous if the target of the exposure is, instead of man, a mouse or a rat. Obviously, the difference between man and a mouse or a rat is great; we are not trying to mimic the "natural conditions" in that part of the experiment where we are using an entirely different anatomical, physiological, and reactive system, such as a small laboratory animal, and trying to interpret it in terms of human exposure. The problem is really one of balance, and we must always ask ourselves why we want to do a certain experiment. We must use experiments as experiments, as guidelines, as exaggerated models, to develop our ability to understand and measure certain types of effects.

In this respect, the importance of positive controls in demonstrating the ability of the animal to yield the desired type of effect, under conditions of maximal challenge, is an extremely important prerequisite that should not be underestimated.

PART II. BENZPYRENE RETENTION STUDIES WITH AN INTRATRACHEAL INSTILLATION MODEL

Some studies conducted in my former laboratory at the Division of Oncology, Institute for Medical Research, The Chicago Medical School, may serve as examples of the approaches needed for the development of animal experimental models for the study of factors involved in the induction of respiratory tumors. The methodology used consists essentially of intratracheal instillation of a saline suspension of a particulated material (e.g., a metal oxide) to which fine particles of a carcinogenic chemical can be attached. Most studies conducted so far have made use of a suspension of benzo[*a*]pyrene (BP) and ferric oxide. The main findings have been summarized in a separate paper for this conference[6] and reported in previous publications.[1,7,14,17,25,26]

Briefly, the mixed dust penetrates into the wall of the respiratory bronchioles and alveoli, where it is phagocytized by macrophages and retained for long periods of time. The soluble carcinogen, however, is rapidly eluted out of the carrier dust and is found to diffuse through the entire architecture of the lung, reaching the epithelium of the large bronchi and trachea by diffusion from the connective tissue stroma.

Tumors develop mostly from the main and segmental bronchi, and they are most frequently squamous cell carcinomas, comparable to those seen in human pathology.

One of the critical problems in the interpretation of the pathogenesis of the induced tumors is represented by the role of the insoluble carrier particles.

The carcinogenic hydrocarbon is administered as a suspension of crystalline particles. Until recently, we were unable to prepare a stable suspension of BP without carrier particles, so in all studies we used a sample of ferric oxide as a constant carrier. Laskin[27] has recently developed a technique for producing a stable suspension of BP alone, and now we have underway the critical experiment of comparing the carcinogenic effects of a BP suspension, administered by itself, with those of the same suspension in conjunction with the administration of ferric oxide.

A. Effects of Pulmonary Dust Load of Carcinogen Retention

The role of particles on the retention time of BP in the lungs was studied in a series of experiments. The retention rates in hamster lungs of different single doses of a 1:1 mixture of BP and ferric oxide showed that BP is eluted out more rapidly when given in smaller doses.[25,26] When the mixture of BP and ferric oxide was given only once, or when it was given for 10 times at weekly intervals, the retention rate for BP remained unchanged, indicating no adaptation in the retention mechanisms.[25,26] In such an experiment, 80–90% of the BP is eluted out of the lung at each week, before the subsequent administration; however, most of the ferric oxide particles, which are not soluble, are retained in the lung. Therefore, at the end of 10 weeks of treatment the animals have a dose of ferric oxide in their lung almost 10 times higher than the animals receiving it only once, yet the retention rate of the soluble BP is practically the same in both groups. This finding was taken to show that an excess amount of dust in the lung does not influence the retention of the soluble carcinogen.

In order to confirm this observation, the following experiments were conducted. The materials used and the general procedures were those previously described.[7,25] A 1:1 mixed dust of BP and ferric oxide was prepared by grinding, analyzed, and kept dry. Individual doses were weighed out on a Cahn electrobalance and put into a 0.5-ml test tube. Saline was added to the tube and the suspension was shaken and taken up with a 0.25-ml syringe fitted with a 19-gauge blunt bent cannula. The cannula was then gently inserted into the trachea of an anesthetized hamster and the suspension instilled. All of the instruments contaminated by the dust were then extracted with acetone and the amount of BP not injected into the animal was determined spectrophotometrically. By difference from the original weight, the exact amount of BP that was retained in the animal at 0 time was determined.

The recovery methods consisted in killing the animals at chosen time intervals in groups of at least four animals, removing their whole lungs and fragmenting them in acetone, then homogenizing the tissue, extracting it with acetone for 4 hr in a Soxhlet apparatus, drying it and then redissolving in a known volume of iso-octane for UV spectrophotometry.

For determinations involving lower levels of recovery, below 10 μg BP, tissue lipids were found to interfere with the spectrophotometric determination. Therefore, all the lung extracts were fractionated by paper chromatography. Each extract was dissolved in iso-octane, evaporated to a few drops under nitrogen and then applied to an 11-cm-wide strip of Whatman No. 1 paper soaked in *n,n*-dimethylformamide (samples with larger doses required up to three strips of paper). Descending chromatography was used with iso-octane as the mobile phase. The BP portion of the chromatogram was located with UV eluted with iso-octane/ethanol (50:50), evaporated to dryness, and redissolved in a known volume of iso-octane; the absorbance was measured at 468 mμ in a Beckman DU spectrophotometer.

Different groups of hamsters received the same measured amounts of BP combined with increasing amounts of ferric oxide dust, by single intratracheal instillation in 0.2 mg saline. The doses tested were: 2 mg of BP added to 2, 4, 6, or 10 mg of hematite. The retention of BP was measured up to 21 days after the administration. Two sets of experiments gave the results illustrated by Figs. 1 and 2.

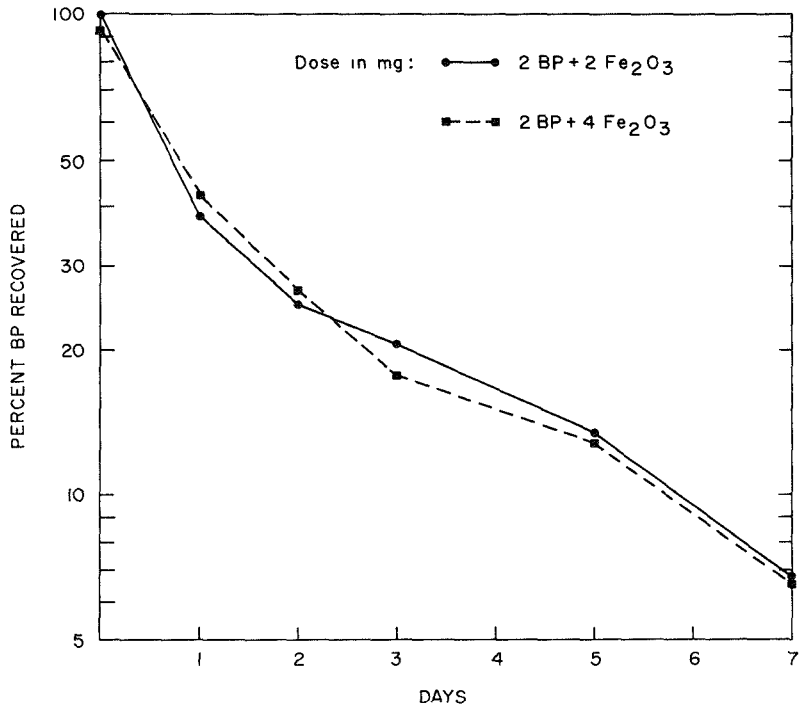

Fig. 1 – Retention of BP in hamster lungs after single intratracheal administration in saline of one of two samples of mixed dusts containing, respectively, 2 mg BP and 2 mg ferric oxide or 2 mg BP and 4 mg ferric oxide.

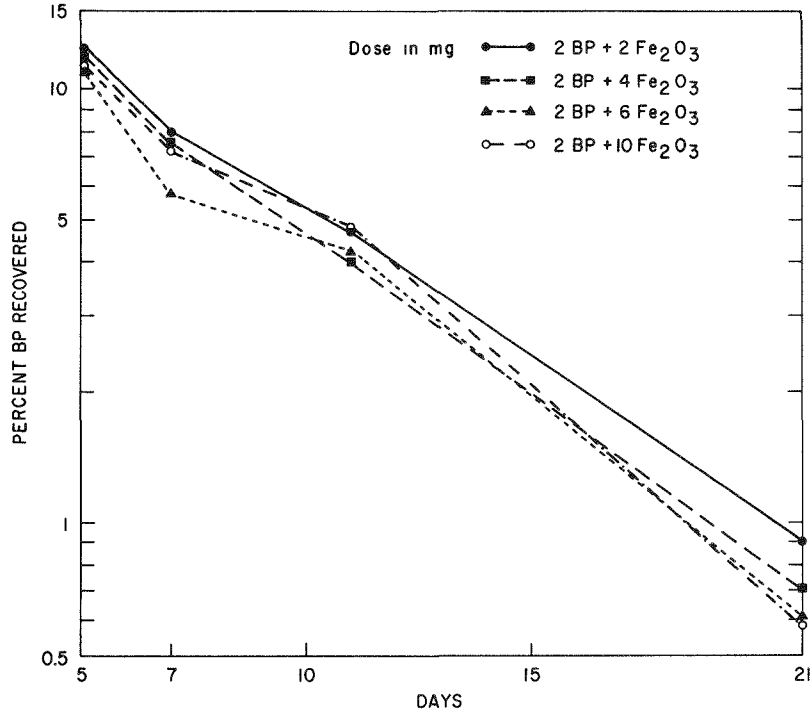

Fig. 2 – Retention of BP in hamster lungs after single intratracheal administration in saline of one of four samples of mixed dusts, all containing 2 mg BP and 2, 4, 6, and 10 mg ferric oxide, respectively.

It is then concluded that, in the experimental conditions so far studied, the retention rate of the carcinogen remains the same in the presence of increasing amounts of dust.

B. Procedures for Preparing Mixed Dust Samples

The samples of BP and ferric oxide used in our studies until 1967 were prepared by grinding, in a mullite mortar, equal weights of BP and ferric oxide. The ferric oxide sample had a known particle size distribution, mostly below 1 μ and BP was obtained in coarse needles. After prolonged grinding, the brittle crystals of BP were reduced approximately to the same size as those of ferric oxide, as shown by microscopic measurement.[7]

Subsequent observations in my laboratory showed that more homogeneous mixtures of a polynuclear hydrocarbon and a carrier dust can be obtained by the following preparation procedure. A concentrated solution of the hydrocarbon in a volume of acetone is poured slowly into 50 volumes of distilled water, containing the dispersed carrier dust at about 4°C on a magnetic stirrer. The

carcinogen then crystallizes into very fine particles attached to the particles of the carrier dust. The particle size of the aggregates of BP and ferric oxide appears dependent on the volume and temperature of the water. The mixed dust suspension is then filtered through a Whatman No. 3 paper on a vacuum flask; the residue is washed off the filter paper with distilled water at room temperature, refiltered, and dried under nitrogen. Aliquots are resuspended in saline when needed. Microscopic control of particle size is necessary. The recovery rate of BP from hamster lungs after instillation of a suspension prepared by grinding was compared with that from a suspension prepared by precipitation from acetone. The two samples contained, respectively, 50.7% and 48.6% BP by weight and had approximately the same particle size distribution.

The recovery rate of BP after administration of a single dose of 4 mg of mixed dust was the same in both cases (Fig. 3). Thus, the method of preparation of the BP crystals does not change its retention in the lungs.

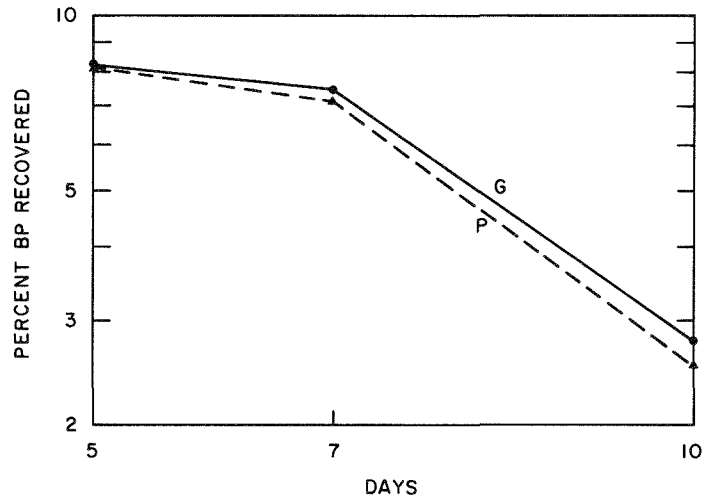

Fig. 3 – Retention of BP in hamster lungs after single intratracheal administration in saline of one of two samples of mixed dust of similar particle size, both containing 2 mg BP and 2 mg ferric oxide, one prepared by grinding and the other prepared by precipitation from acetone.

C. Effects of Particle Size on Carcinogen Retention

The BP retention rates in hamster lungs were determined for three samples of mixed dusts having the same composition (BP and ferric oxide 1:1) but different particle sizes (Fig. 4). Dust A was prepared by grinding, and contained particles up to 17.5 μ. Dust B was prepared by precipitation from acetone and contained particles up to 12 μ. Dust C was obtained by the same method

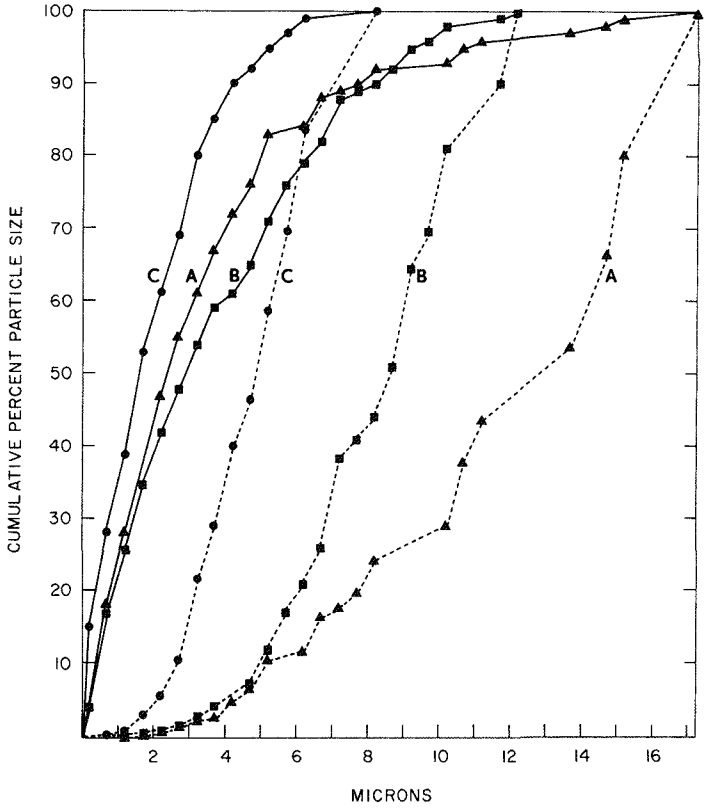

Fig. 4 – Particle size distribution by number (full lines) and by weight (dotted lines) of three samples of mixed dusts containing BP and ferric oxide 1:1 by weight. Dust A obtained by grinding; dust B obtained by precipitation from acetone, dust C obtained by precipitation from acetone followed by ultrasonic treatment.

followed by ultrasonic treatment to break up the larger particles: its maximum particle size only reached 8.2 μ. A single 4 mg dose of each dust (2 mg BP + 2 mg ferric oxide) was administered to young adult male Syrian golden hamsters and BP retention rates were determined on at least four animals per point. The results, given in Fig. 5, indicate that the percent retention of BP in the lung decreases particle size.

These experiments are of a preliminary nature. A more detailed characterization of the physico-chemical properties of the particles and a broader spectrum of sample types are needed to define the entity of this effect. The results so far obtained are suggestive of a marked effect of the particle size distribution on the retention of BP in the lung, while no variation of retention rate occurs when the total ferric oxide load is increased up to 10 times.

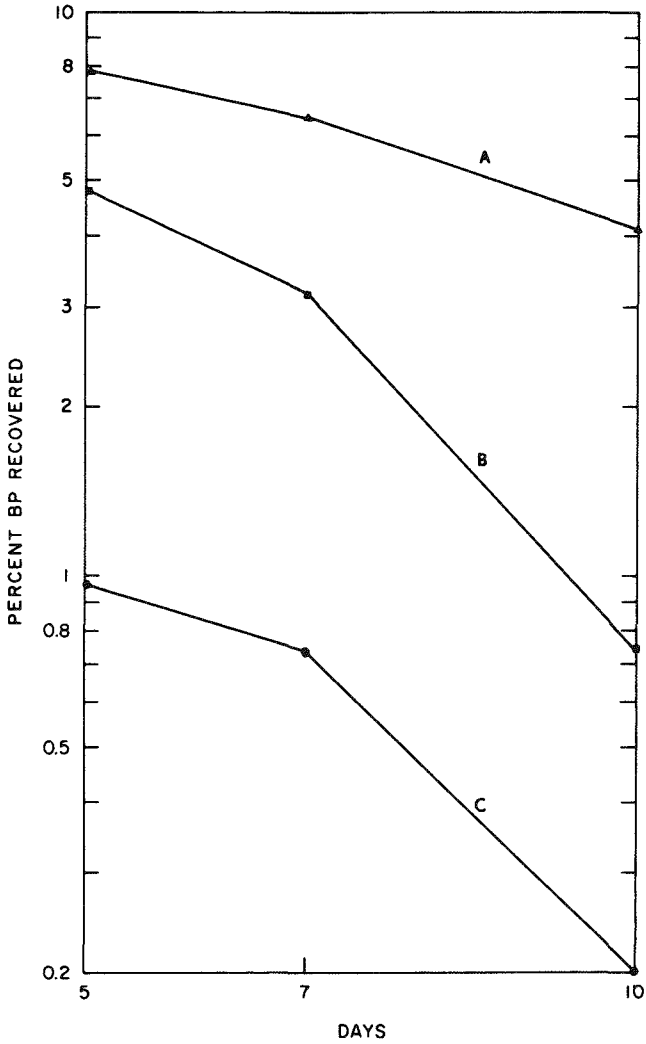

Fig. 5 – Retention of BP in hamster lungs after a single intratracheal administration in saline of a sample of dusts A, B, and C, each containing 2 mg BP and 2 mg ferric oxide. (See legend to Fig. 4 for description of dusts.)

PART III. MORPHOLOGICAL AND BIOCHEMICAL CORRELATIONS: THE PROBLEM OF DIFFERENTIATION

A. Morphological Analysis of Induced Respiratory Tumors

The studies on respiratory carcinogenesis conducted in our laboratories in the past decade are reviewed separately in this conference.[6] An analysis of the

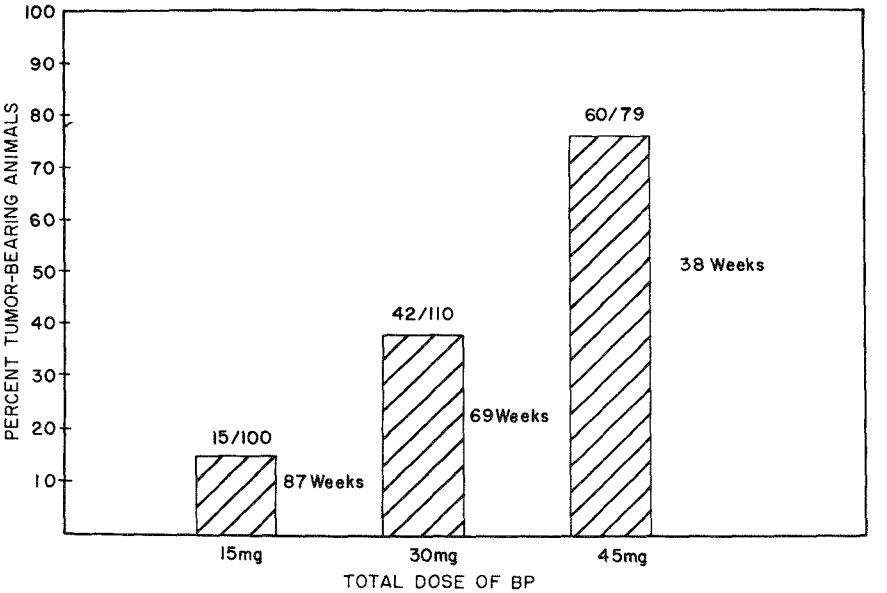

Fig. 6 – Respiratory tumors induced by BP and ferric oxide in hamsters. The three dose levels were obtained with 5, 10 or 15 intratracheal instillations of a dose of 3 mg BP and 3 mg ferric oxide in 0.2 ml saline. Figures on each column represent the numbers of tumor-bearing animals over the total number of animals autopsied. The average latent period for all tumors is given in weeks.

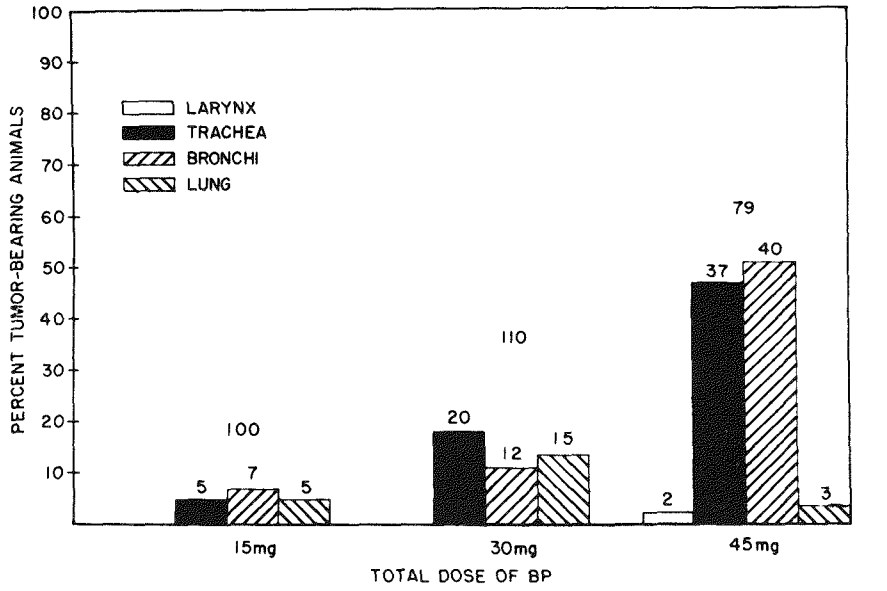

Fig. 7 – Same experiment as in Fig. 6. Distribution of tumors in each segment of the respiratory tract. The figures on each column represent the total number of tumors at each site; those above, the total number of animals autopsied.

induced tumors by organ sites and by main histologic differentiation types is reported here for two typical experimental models.

In one series of experiments[7,17] a mixed dust, containing equal weights of BP and ferric oxide particles suspended in saline, was administered intratracheally. Each dose was of 3 mg BP + 3 mg Fe_2O_3 saline. Two groups of hamsters received 15 doses, 2 groups received 10 doses and 2 groups received 5 doses. All doses were given at weekly intervals (except for one of the groups at 5 doses, which received them at intervals of 25 days). For the purpose of this analysis, the results obtained in the two groups at each dose level are pooled. The total number and average latent period of induced respiratory tumors, as given in Fig. 6, demonstrate the dose-response correlations. The distribution of the tumors by site of origin in the various segments of the respiratory tract (Fig. 7) shows a low response in the larynx only at the highest dose, a positive dose response correlation for the induction of tumors in the trachea and in the bronchi, but only a low incidence at all dose levels for peripheral pulmonary tumors. A cumulative analysis of all tumors for their differentiation (Fig. 8) shows that adenomatous tumors (or tumors with mucous differentiation) remain low at all dose levels, whereas anaplastic and squamous tumors increase with increasing doses, the squamous tumors reaching the highest incidence. The detailed morphology of the tumors has been previously illustrated.[7]

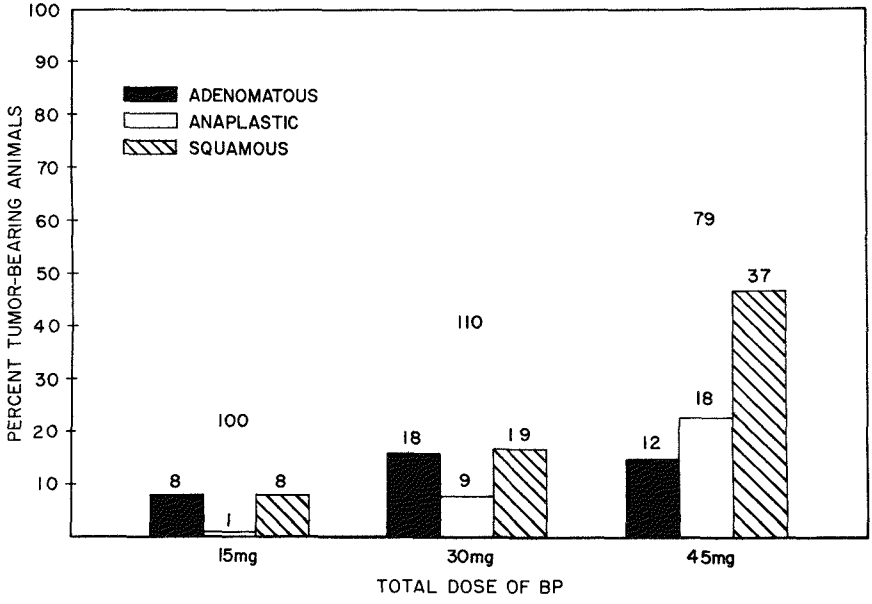

Fig. 8 – Same experiment as in Figs. 6 and 7. Distribution of all respiratory tumors by histologic type of differentiation. The figures on each column represent the total number of tumors of each type; those above, the total number of animals autopsied.

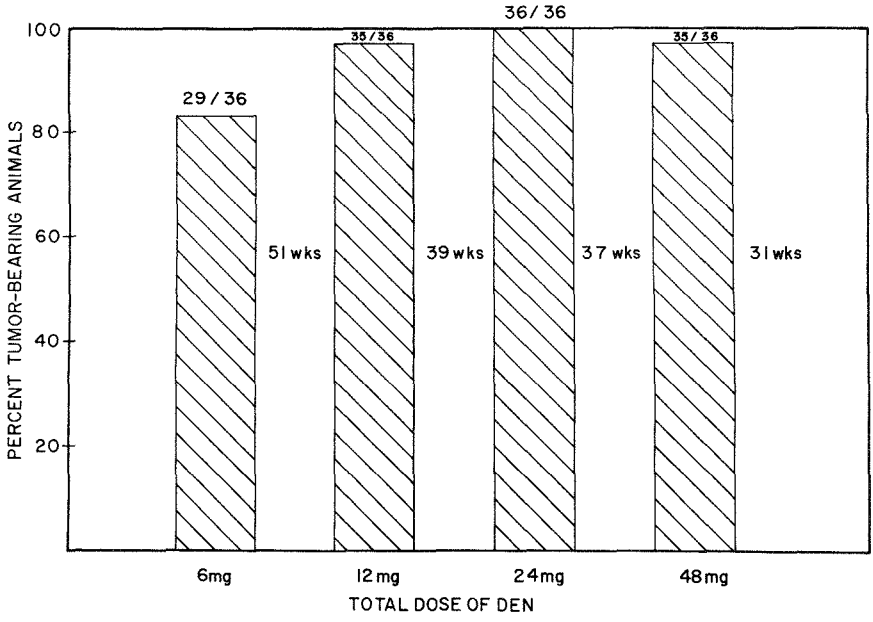

Fig. 9 – Respiratory tumors induced by DEN administered subcutaneously to adult hamsters. The four dose levels were obtained with 12 weekly subcutaneous injections of 0.5, 1.0, 2.0 or 4.0 mg DEN. Figures on each column represent the numbers of tumor-bearing animals over the total number of animals autopsied. The average latent period for all tumors is given in weeks.

Treatment of Syrian golden hamsters with different doses of the systemic carcinogen diethylnitrosamine (DEN) has resulted in an entirely different pattern of tumor response. When DEN was administered weekly to four groups of young adult hamsters in 12 doses of 0.5, 1.0, 2.0 and 4.0 mg, respectively,[8] a high tumor yield was obtained in all groups (Fig. 9). The distribution by site (Fig. 10) shows a high frequency of tumors in the nasal cavities, which was never seen after BP treatment. The larynx was affected with increasing frequency at increasing doses, and the trachea was always the segment most highly involved in the tumor response. A minimal response was detected in the bronchi and lungs. An analysis of tumor differentiation types (Fig. 11) reveals a large incidence of papillary tumors, listed as polyps or papillomas, with varying degrees of differentiation – from columnar mucous cells to squamous cells. Very few tumors were anaplastic or frankly squamous. The morphology of DEN-induced tumors has been illustrated in detail.[8] A similar pattern was found in hamsters that had received DEN at birth at different dose levels,[19] except that under these conditions the incidence of tumors of the nasal cavities remains relatively low (Figs. 12, 13, and 14).

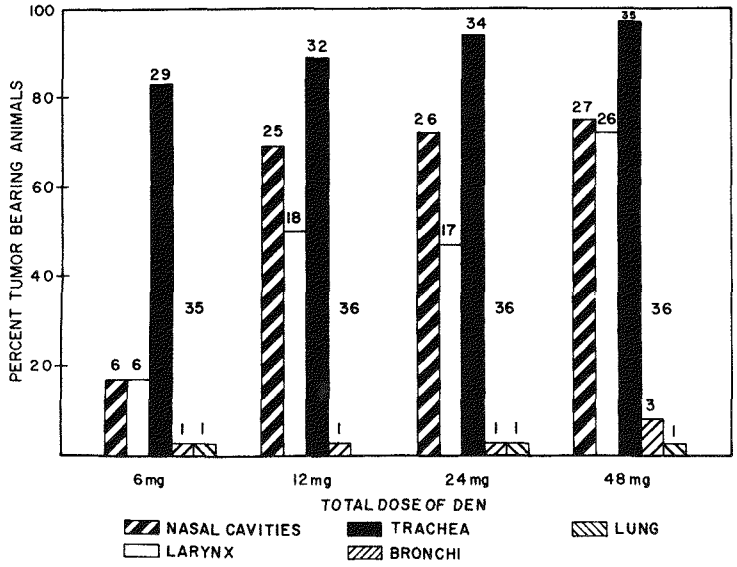

Fig 10 – Same experiment as in Fig 9 Distribution of tumors in each segment of the respiratory tract The figures on each column represent the total number of tumors at each site, the figures on the side, the total number of animals autopsied

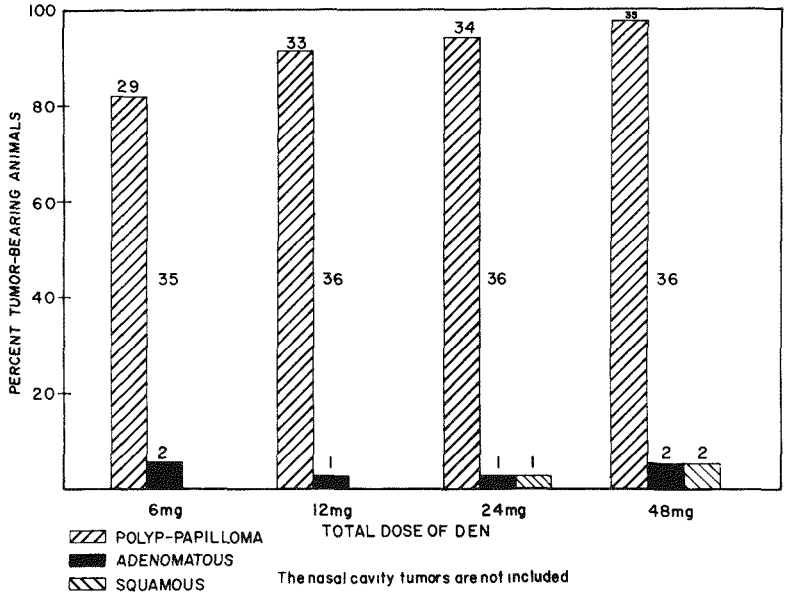

Fig 11 – Same experiment as in Figs 9 and 10 Distribution of all respiratory tumors by histologic type of differentiation The figures on each column represent the total number of tumors of each type, the figures on the side, the total number of animals autopsied

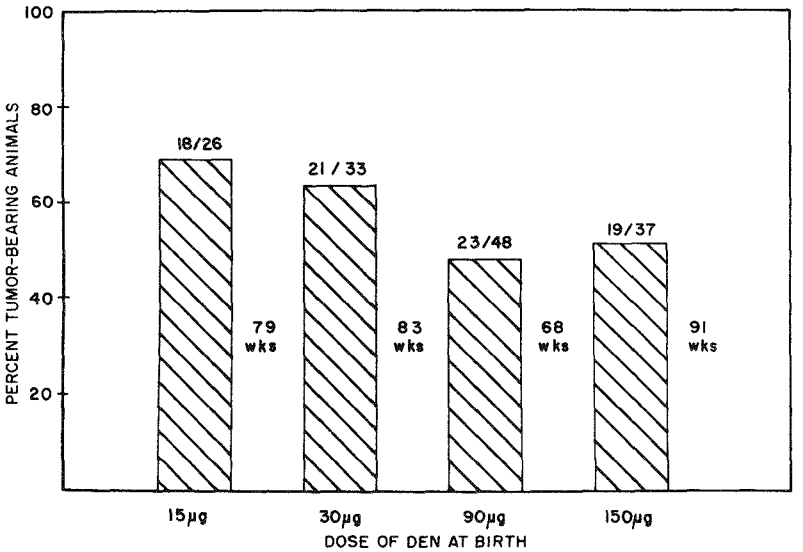

Fig 12 – Respiratory tumors induced by DEN administered subcutaneously to newborn hamsters Percent tumor-bearing animals (T B A) at four dose levels given as a single subcutaneous injection Figures on each column represent the numbers of tumor-bearing animals over the total number of animals autopsied The average latent period for all tumors is given in weeks

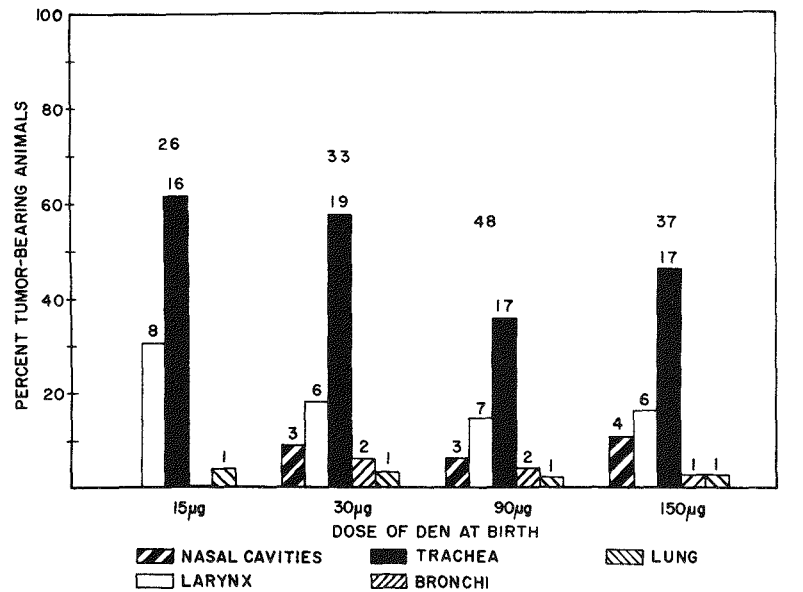

Fig 13 – Same experiment as in Fig 12 Distribution of tumors in each segment of the respiratory tract The figures on each column represent the total number of tumors at each site, those above, the total number of animals autopsied

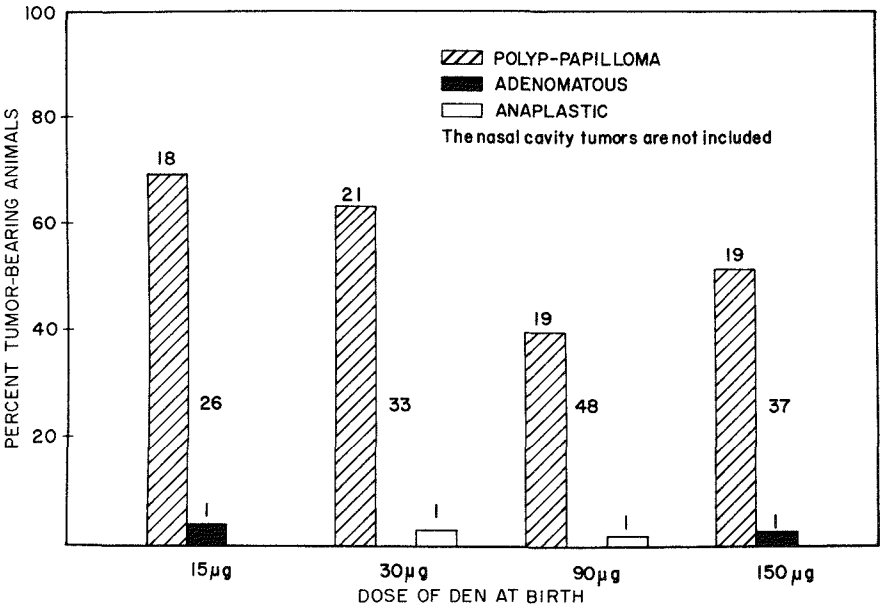

Fig. 14 – Same experiment as in Figs. 12 and 13. Distribution of all respiratory tumors by histologic type of differentiation. The figures on each column represent the total number of tumors of each type; the figures on the side, the total number of animals autopsied.

More extensive morphological analyses on the tumors obtained by the variety of experimental models developed by different laboratories could be useful in assessing the quality and specificity of the neoplastic response of the respiratory tract in various species and under various experimental conditions. Such information would be of particular value in our attempt to define the experimental models to be used for studies of respiratory carcinogenesis and to correlate the morphological end points with pathogenetic factors and with their counterparts in man.

B. Cellular Differentiation in Respiratory Carcinogenesis

The majority of bronchogenic carcinomas in man are characterized by a squamous differentiation. We have reproduced the same response in animals by administration of polynuclear hydrocarbons on a particulated carrier. In these circumstances, the respiratory epithelium undergoes a dual change: (1) metaplasia from a columnar mucous-synthetizing epithelium to a squamous-keratinizing epithelium; and (2) transformation from a normal to a neoplastic state.

We have reported earlier[15,28] that in the hamsters intratracheally treated with BP, the subsequent oral administration of vitamin A palmitate at high doses sharply inhibits both effects, resulting in a block of squamous differentiation as well as a marked reduction of tumor incidence. A marked inhibition was observed concurrently in the development of squamous tumors of the forestomach.

Vitamin A was selected for this study because of its role in the control of differentiation. The fact that it also controlled the development of tumors suggested that a common mechanism may be involved in the two effects.[29] The implications of this hypothesis are far reaching. They extend from the study of cellular differentiation as a basic aspect of the biology of carcinogenesis, to the identification of host susceptibility factors required for the carcinogenic response in the respiratory tract (e.g., tissue levels of retinol), to the possibility that preventive measures can be developed for the control of squamous cell cancers in man.

Little is known of the molecular mechanisms by which vitamin A directs the synthesis of specific proteins characterizing the states of differentiation controlled by it, namely, glycoproteins and keratin. An important contribution has recently been provided by the studies of De Luca and Wolf,[30,31] who have undertaken an analysis of the pathways of protein synthesis in normal and vitamin A-deficient animals, using the rat intestinal mucosa as their model tissue. Their results demonstrate that protein synthesis by membrance-bound but not by free polyribosomes of intestinal epithelium is depressed under conditions of vitamin A deficiency. Therefore, they conclude that vitamin A – in intestinal mucosa and, by extension, most probably in other mucosa epithelia – functions at some point in the process of protein synthesis, either directly or indirectly at the translational level. The evidence obtained shows that one of the glycoproteins of intestinal mucosa is not synthetized in vitamin A deficiency. The lesion caused by the deficiency is located in the pH 5 fraction and not in the rough endoplasmic reticulum. The results so far obtained[31] point to an effect of vitamin A deficiency on the aminoacyl tRNA synthetase enzymes. DeLuca and Wolf,[31] considering the growing evidence in favor of a translational control of differentiation, speculate that to change the type of protein to be translated, a new set of synthetases has to be activated or made to match a change in the tRNA that can recognize a change in the messenger RNA. They suggest that perhaps vitamin A can influence the type of aminoacyl tRNA synthetase activity or biosynthesis in mucosal cells, thereby controlling differentiation.

There is now an increasing number of studies concerning the role of translational control mechanisms and particularly the role of tRNA in the process of carcinogenesis. The hypothesis can be advanced that a common pathway may control differentiation and carcinogenesis, and that it may explain the observed simultaneous inhibition by vitamin A of squamous differentiation

and of carcinogenesis in the respiratory epithelium. We have initiated a series of studies aimed at elucidating this problem, in collaboration with Drs. Wolf and De Luca.

The suggested role of vitamin A in controlling carcinogenesis in the respiratory tract – and probably in other mucous epithelia – is corroborated by other observations. Using *in vitro* organ cultures of newborn hamster trachea, Crocker[32] has recently demonstrated the inhibition by vitamin A of the metaplastic changes induced by BP in the tracheal epithelium.

We have recently examined the vitamin A content of different, currently used animal diets. To our surprise, we found that the diet we had used for all our respiratory carcinogenesis studies in hamsters (Rockland Rat Diet A. E. Staley Mfg. Co., Decatur, Ill.) had the lowest vitamin A content (about 600 USP units/100 g of all those examined; six other diets had vitamin A contents varying from 1200 to 2200 USP units/100 g). Whether or not such a low level of vitamin A in the diet was a contributory factor to the high yield of squamous tumors obtained in our experiments remains to be determined by properly controlled comparative carcinogenesis tests conducted at different levels of vitamin A intake.

Recent studies in man, in which retinol levels were determined in the liver[33] or in the serum,[34] revealed the unexpected fact that about one-third of the population examined had very low vitamin A levels. A study could be conducted to determine whether individuals with a particularly low retention of vitamin A represent a high risk group for certain types of cancers, such as bronchogenic squamous cell carcinoma.

PART IV. CONCLUSIONS

The papers to be presented at this conference will review the main experimental models for the induction of respiratory tumors in animals. Methods involving a direct and fairly strong exposure of the tracheobronchial mucosa to chemical and physical carcinogens have been those most successful in reproducing in the experimental animals lung cancer types as we see them in man.

Experimental models such as those developed by the group at New York University,[9,10,35] by Shabad and Pylev in Moscow,[11-13] and by our former group at Chicago[1,6,7,14] have been most useful in demonstrating the susceptibility of experimental animals to the induction of bronchogenic carcinoma by chemicals.

Inhalation exposures have been repeatedly successful with exposure to radionuclides.[36] On the other hand, only relatively few instances of respiratory carcinogenesis by inhaled chemicals have been experimentally well documented (e.g., nickel carbonyl, beryllium, asbestos, certain *N*-nitroso compounds).

Suggested reasons for the lack of success of many other attempts at inhalation carcinogenesis have been given in the first part of this paper

Inhalation exposures of animals to tobacco smoke failed in the past to given direct evidence of experimental induction of respiratory tract carcinomas The reasons for this failure are again those discussed above, mainly the insufficient dose at the target site It is therefore extremely important that a positive result has been obtained by Dontenwill[37] with the induction of laryngeal carcinomas in hamsters directly exposed to tobacco smoke

The study of Auerbach in dogs[38] is about to enter the critical time period when bronchogenic carcinoma, resulting from the direct exposure to cigarette smoke, can be expected to occur

The recent finding that inhalation of SO_2, combined with exposure to BP, determines the induction of bronchogenic carcinoma in rats[10,35] represents a remarkable step forward in the evaluation of the role played by factors that do not appear related to the carcinogenesis when tested alone Another demonstration of this type of effect is provided by our recent finding[6,18] that intratracheal instillations of ferric oxide strikingly increase the carcinogenic response to DEN in the bronchopulmonary tissues

Those preliminary observations suggest that combined treatments may become useful experimental devices to reveal certain types of carcinogenic effects

Pretreatments with known carcinogens, acting as a sort of priming treatment, may be able to raise considerably the susceptibility of the animal target systems, so that they might become sensitive to low levels of exposure to inhaled materials More experimentation is needed along these lines

Another important consideration concerns biochemical studies of the binding or interaction of chemical carcinogens with target-cell constitutents in the respiratory tract for the purpose of correlating them with carcinogenesis on a quantitative basis

We know that the interaction will occur within a short period of time, as in the case of DEN at the level of the bronchopulmonary tissues, and a very low incidence of tumors develops if no other treatment is added Yet, a subsequent exposure to as simple a material as ferric oxide can generate a 70% incidence of tumors, thus indicating that the critical binding of the carcinogen to the target-cell constitutent had already taken place, even if it had not become apparent without the secondary treatment

In concluding this discussion of experimental problems related to inhalation carcinogenesis, I think that we could say that the definition of the host response to multiple, interacting pathogenetic factors can be used to develop animal models capable of highly sensitive, selective carcinogenic responses to respiratory exposures, even at relatively low dose levels, such as those that are provided by inhalation techniques

ACKNOWLEDGMENTS

I wish to thank Drs R Montesano and A R Sellakumar for their collaboration in the studies reported in Parts II and III, and Dr M Glassman for reviewing data on vitamin A content in animal diets Experimental work was carried out at the Division of Oncology, Institute of Medical Research, The Chicago Medical School until April 1968 (supported by USPHS Research Career Development Award K3-CA-25027 from the National Cancer Institute, NIH, and by Research Project Contract MC-6 from the American Petroleum Institute) The work was then performed in part at The Eppley Institute for Research in Cancer, University of Nebraska College of Medicine, Omaha (supported by Contract PH43-68-959 from the National Cancer Institute, NIH, USPHS)

REFERENCES

1 U Saffiotti, Experimental respiratory tract carcinogenesis In, *Prog Exptl Tumor Res* Vol II, pp 302–333, F Homburger (ed) Karger, New York/Basel, 1969
2 U Saffiotti and C G Baker, Program planning in inhalation carcinogenesis NCI plans In, these *Proceedings* p 467
3 N Nelson, Inhalation carcinogenesis in man Environmental and occupational hazards In, these *Proceedings,* pp 3–12
4 H L Falk, Chemical definitions of inhalation hazards In, these *Proceedings,* pp 13–26
5 L Kreyberg, A A Liebow, and E A Uehlinger, *Histological Typing of Lung Tumours,* International Histological Classification of Tumours, No 1 World Health Organization, 1967
6 R Montesano, U Saffiotti, and P Shubik, The role of topical and systemic factors in experimental respiratory carcinogenesis In, these *Proceedings* pp 353–371
7 U Saffiotti, F Cefis, L H Kolb, A method for the experimental induction of bronchogenic carcinoma *Cancer Res* 28 (1968) 104–124
8 R Montesano and U Saffiotti, Carcinogenic response of the respiratory tract of Syrian golden hamsters to different doses of diethylnitrosamine *Cancer Res*, 28 (1968) 2197–2210
9 M Kuschner, S Laskin, E Cristofano, and N Nelson, Experimental carcinoma of the lung In, *Proc 3rd Natl Cancer Conf*, pp 485–495 Lippincott, Philadelphia, 1956
10 M Kuschner, The J Burns Amberson lecture The causes of lung cancer *Am Rev Respirat Diseases,* 98 (1968) 573–590
11 L N Pylev, Induction of lung cancer in rats by intratracheal insufflation of cancerogenic hydrocarbons *Acta Unio Intern Contra Cancer,* 19 (1962) 688–691
12 L M Shabad, Experimental cancer of the lung *J Natl Cancer Inst*, 28 (1962) 1305–1332
13 L M Shabad, Pathology and pathogenesis of experimental lung tumours with reference to human lung cancer prevention In, *Lung Tumours in Animals* Proc 3rd Quadrennial Intern Conf Cancer, pp 85–100, L Severi (ed) Div of Cancer Res , Univ of Perugia, Italy, 1966
14 U Saffiotti F Cefis, and P Shubik, Histopathology and histogenesis of lung cancer induced in hamsters by carcinogens carried by dust particles In, *Lung Tumours in Animals* Proc 3rd Quadrennial Intern Conf Cancer, pp 537–546, L Severi (ed) Div of Cancer Res Univ of Perugia, Italy, 1966

15. U. Saffiotti, R. Montesano, A. R. Sellakumar, and S. A. Borg, Studies on experimental lung cancer: Inhibition by vitamin A of the induction of tracheobronchial squamous metaplasia and squamous cell tumors. *Cancer,* 20 (1967) 857–864.
16. U. Saffiotti, R. Montesano, and A. R. Sellakumar, Correlation between the histogenesis of respiratory tract tumors and their mechanisms of induction. *Am. J. Pathol.,* 52 (1968) 59a–60a.
17. U. Saffiotti, R. Montesano, A. R. Sellakumar, and F. Cefis, Respiratory tract carcinogenesis in hamsters induced by different numbers of administrations of benzo[*a*]pyrene and ferric oxide. *Cancer Res.,* in press, 1970.
18. R. Montesano, U. Saffiotti, and P. Shubik, Synergistic effects of diethylnitrosamine, benzo[*a*]pyrene and ferric oxide on respiratory carcinogenesis in hamsters. *Cancer Res.,* in press, 1970.
19. R. Montesano, U. Saffiotti, and P. Shubik, Carcinogenic response of hamster respiratory tract to single subcutaneous administrations of diethylnitrosamine at birth. *J. Natl. Cancer Inst.,* 44 (1970) 413–417.
20. W. Dontenwill and U. Mohr, Carcinome des Respirationstractus nach Behandlung von Goldhamstern mit Diäthylnitrosamin. *Z. Krebsforsch.,* 64 (1961) 305–312.
21. W. Dontenwill, U. Mohr, and M. Zagel, Uber die Unterschiedliche Lungencarcinogene Wirkung des Diäthylnitrosamin bei Hamster und Ratte. *Z. Krebsforsch.* 64 (1962) 499–502.
22. K. M. Herrold and L. J. Dunham, Induction of carcinoma and papilloma of the tracheobronchial mucosa in the Syrian hamster by intratracheal instillations of benzo[*a*]pyrene. *J. Natl. Cancer Inst.,* 28 (1962) 467–491.
23. K. M. Herrold and L. J. Dunham, Induction of tumors in the Syrian hamster with diethylnitrosamine (N-nitrosodiethylamine). *Cancer Res.,* 23 (1963) 773–777.
24. K. M. Herrold, personal communication.
25. U. Saffiotti, S. A. Borg, M. I. Grote, and D. A. Karp, Retention rates of particulate carcinogens in the lungs, studies in an experimental model for lung cancer induction. *The Chicago Medical School Quart.,* 24, No. 2, (1964) 10–17.
26. U. Saffiotti, F. Cefis, L. H. Kolb, and P. Shubik, Experimental studies of the conditions of exposure to carcinogens for lung cancer induction. *J. Air Pollution Control Assoc.,* 15 (1965) 23–25.
27. S. Laskin, personal communication.
28. U. Saffiotti, R. Montesano, and A. R. Sellakumar, Further studies on inhibition by vitamin A of respiratory tract carcinogenesis. *Federation Proc.,* 26 (1967) 692.
29. U. Saffiotti, Role of vitamin A in carcinogenesis. *Am. J. Clin. Nutr.,* 22 (1969) 1088.
30. L. De Luca, E. P. Little, and G. Wolf, Vitamin A and protein synthesis by rat intestinal mucosa. *J. Biol. Chem.,* 244 (1969) 701–708.
31. L. De Luca and G. Wolf, Vitamin A and protein synthesis in mucous membranes. *Am. J. Clin. Nutr.,* 22 (1969) 1050–1062.
32. T. T. Crocker, Effect of vitamin A *in vitro,* in press, 1970.
33. K. Hoppner, W. E. J. Phillips, T. K. Murray, and J. S. Campbell, Survey of liver vitamin A stores in Canadians. *J. Can. Med. Assoc.,* 99 (1968) 983–986.
34. A. E. Schaefer, Hidden hunger. *Med. World News,* 10 (1969) 17–19.
35. S. Laskin, M. Kuschner, and R. Drew, Studies in pulmonary carcinogenesis. In, these *Proceedings,* pp. 321–351.
36. W. J. Bair, Inhalation of radionuclides and carcinogenesis. In, these *Proceedings,* p. 77.
37. W. Dontenwill, Experimental investigations on the effect of cigarette smoke inhalation on small laboratory animals. In, these *Proceedings,* pp. 389–412.
38. O. Auerbach, Emphysema produced in dogs by cigarette smoking. In, these *Proceedings,* pp. 375–387.

DISCUSSION

W. Dontenwill: I think the stay period of carcinogens in the lung is very important for the carcinogenic activity. We have seen different reactions after intertracheal injection of DMBA into the lungs of hamsters, first in a real solution with sesame oil and then in a suspension with carboxymethyl cellulose. The stay period in a real solution is 2 days, and the stay period in suspension is approximately 22 days.

U. Saffiotti: Yes. The whole problem of trying to determine the characteristics of the retention of carcinogens is very important, and to change the ability of the tissue to retain a carcinogen may be again a key to enhancing the effective exposure. It will be very important to define the role of vehicles and solvents in determining retention of carcinogens.

E. P. Radford: Dr. Saffiotti's data showed a very strong dependence of the latent period on dose, whereas in human beings (from the available data on mining populations such as Dr. Nelson showed earlier this morning) there doesn't seem to be such a strong dependence. Would you comment on this?

Saffiotti: I think that the problem is more complex than any single method of analysis can show. Survival rate, time of death of animals with tumors of each type, total number of tumors, and various indices of tumor yield have to be evaluated together.

We do find that in our system of respiratory carcinogenesis, as in many others (e.g., carcinogenesis of the skin, subcutaneous regions, liver), there is not only a correlation of dose with total yield of tumors but also an inverse relation to the latent period.

In humans, this is perhaps reflected to some extent in the shortening of the average time of death by lung cancer in cohorts. The older cohorts reach given incidences at a later age than the younger cohorts.

Radford: How easily can you carry over data from animal experiments to humans?

Saffiotti: Well, that obviously is the critical point – it's a long story!

F. G. Bock: I've talked about this problem to some epidemiologists, and it seems that when you are dealing in an experimental system with a fairly high incidence, a latent period shift would be expected with an increase of dose; whereas in the human situation, where you are dealing with a very low incidence, a latent period shift is not necessarily expected when you increase the dose, because you have a tremendous pool of unaffected people.

Saffiotti: I think this goes back to our need for better human data, and it may be that in lung cancer, where the incidence is so high, we will eventually have enough data to analyze some of these variables quite precisely in humans.

K. H. Kilburn: I wish to raise one general question that I think is pertinent and bothers all of us who use model systems: Are there any ways in which we can extrapolate to man from the dimensions of the lung in a 100-g hamster? Tumors occur in the hamster in the larynx, trachea, and peripherally in either segmental or terminal bronchi, or bronchioles – indeed in the same locations where tumors are found in human lungs. However, structurally analogous airway diameters are practically orders of magnitude different. Somehow it seems that analogies may be fortuitous – which is to say that they reflect compensating differences.

Saffiotti: Obviously, the dimensions, the anatomical structures, and many other aspects of the experimental models are very different from those in men. Yet I think it is important that we get the same overall type of response at the tissue and cellular level. We see the same type of cellular differentiation and the same type of malignancy. In our experiments we get almost the same ratio of squamous to anaplastic to adenocarcinomas that have been reported in man. This comforts us in assuming that some of these systems are meaningful for the type of reaction that occurs in man.

T. T. Crocker: I think this is an important issue. We must be attentive not only to the size of the airways of the various animal species, but also to the cellular responses that Dr. Saffiotti has just referred to as being similar in the living hamster model and in man.

In the organ culture system, we have responses of various animal tissues to hydrocarbons at known concentrations for known periods of exposure. We have found real similarity in cellular and histologic changes among mouse, rat, hamster, dog, and monkey respiratory epithelia if the animals are of about the same age at the time the tissue is taken for explantation. If our living animal models do not respond to carcinogens in a uniform way, it is not necessarily due to differences in the responsiveness of the target tissue. Differences between the toxic or carcinogenic responses of living animals may have to be sought in some location other than the lung. In line with your report of the hamster lung cancer model, we have been exposing hamsters and a prosimian primate (*Galago crassicaudatus*) known as the Bushbaby to benzo[*a*]pyrene and hematite. Responses of the two animals to the same material were similar in the bronchi, but the hamsters did not experience hematologic depressions, while the primates did. This represents a difference between two living animal models for lung carcinogenesis in which metabolic mechanisms remote from the target tissue, such as detoxification of carcinogen after absorption from the lung, may influence the ability of two animal orders to sustain long-term dosage.

M. Kuschner: Dr. Saffiotti, in following up Dr. Dontenwill's question, you didn't show us the disappearance curves for benzo[*a*]pyrene alone. I wonder if you could draw that for us; I don't think it was included.

Saffiotti: No, it was not.

Kuschner: I would like to know the rate of clearance.

Saffiotti: That is now under study. As you know, the problem we faced for several years was that we did not have a practical way to introduce benzo[*a*]pyrene without resorting to surface-active agents or solvents. Laskin has recently prepared such a special suspension by long-term grinding of benzo[*a*]pyrene. With such techniques, one can now compare the clearance and the effects of the polynuclear hydrocarbon apart from those of the dusts.

Kuschner: I would like to say, referring to Dr. Radford's question, that there is perhaps a confirmation of your impression of a shorter latent period at higher doses. Judging from the very high incidence of these tumors found in uranium miners, I think the tumors (which are mostly oat-cell) occur a decade earlier.

Radford: I didn't mean to imply that there was no difference in latent period between high and low radon exposures; but, considering the difference for human subjects between 10 years minimum latent period at the highest doses to perhaps 20 years at the very lowest doses, there is quite a difference from the variation of latent period seen in the animal experiments.

A. P. Wehner: You suggested pretreating the animals with a third agent to lower the threshold of response. If we want to investigate, for instance, the cocarcinogenicity of asbestos and cigarette smoke, and if we pretreat these animals with, say, benzo[*a*]pyrene and hematite, isn't it conceivable that we would then generate an entirely different situation?

Saffiotti: Yes, and one will never have enough controls for experiments of this kind. I think that, eventually, there will be enough work along these lines to define under what conditions we will be able to enhance the response of a given biological target.

EXPERIMENTAL INHALATION STUDIES – EQUIPMENT AND PROCEDURES

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ABSTRACT

The factors that affect the deposition of airborne contaminants in the respiratory tract are reviewed, including the physical and chemical properties of the contaminant and the airway dimensions and respiratory characteristics of the subject exposed. — Design features of systems for experimental inhalation exposures are outlined. This discussion includes delivery techniques (*i e*, nasal tubes, face masks, and chambers for daily and lifetime exposures) Critical parameters affecting chamber performance are reviewed Techniques and equipment for producing and monitoring controlled test atmospheres and for studies of the deposition and translocation of respirable particles are described

Respiratory tract cancer has been produced in laboratory animals exposed to airborne carcinogens in recent years, and some of the studies in which it has been accomplished will be described in Session III of this conference However, most attempts to produce cancers by inhalation have been unsuccessful, due in part to the incomplete knowledge of the conditions necessary to initiate cancer at primary reaction sites In many instances the lack of success was also due to (1) the inability of the investigators to deliver a sufficient dose of the carcinogen to the site of toxic action, because of inadequate knowledge concerning the deposition sites within the respiratory tract for inhaled material, and (2) the limited availability of reliable equipment and procedures for controlled inhalation exposure

This discussion is an attempt to provide some background and guidance on the factors affecting the delivery of airborne contaminants to the zones of the respiratory tract of interest and the techniques and equipment available for this purpose

For particles, regional deposition and clearance dynamics affect the toxicity or carcinogenicity of an inhaled aerosol, since these factors determine the

amount and pattern of topical dose on the respiratory epithelium. The surface of the respiratory tract varies considerably in structure and susceptibility to tissue reaction, as well as in the mechanisms by which deposited particles are removed. The nose, the nasopharynx, larynx, tracheobronchial tree, and alveoli differ from one another in amount of deposition, pattern of deposition, types of epithelial cells, and/or mechanisms for particle elimination.

The distribution of deposition of inhaled contaminants within the respiratory tract is dependent on the physical and chemical properties of the contaminant, and on the anatomical and respiratory characteristics of the animal being exposed. One of the first considerations is the state of dispersion of the contaminant. The motion of contaminant particles through the respiratory tract may differ markedly from that of the carrier airstream. For particles with diameters below ~0.25 μ, most of the deposition will result from the Brownian motion of the particles, and will increase with decreasing diameter. For particles larger than about 0.5 μ (*i.e.*, particles whose motion is largely determined by gravitational and/or inertial forces) the critical parameter is aerodynamic size. This parameter, defined as the diameter of a unit density sphere having the same terminal settling velocity as the particle in question, takes into account the variable linear dimensions, density, and aerodynamic drag factors, which may vary considerably for nonspherical particles.

The electric mobility of the particle, which is determined by its charge-to-mass ratio, may also influence its deposition probability. Although electrostatic deposition is usually relatively unimportant for most ambient aerosols, it may be very important for laboratory-generated aerosols that are inhaled by the exposed animals before coming to charge equilibrium.[1] Another complication is introduced when the particles are hygroscopic, since they will grow in size until they reach an equilibrium determined by water vapor saturation at ambient body temperature.

A particle which strikes the wall of an airway – whether by impaction, interception sedimentation, or in the course of Brownian motion – is considered to be collected with a 100% efficiency. For particles larger than 0.5 μ, most of the deposition in the conductive airways will result from inertial and sedimentation forces; consequently, the distribution of the deposited particles on the walls of conductive airways will not be uniform. In the larger airways, where impaction is the dominant mechanism, the deposited particles will be concentrated on a small fraction of the surface, especially at the bronchial bifurcations. In the smaller conductive airways, where sedimentation is dominant, the particles will deposit on the lower halves of the tubes.

For particles smaller than ~0.25 μ, and for gases and vapors where the dimensions are molecular, the Brownian motion of the contaminant causes surface deposition. Gas molecules may rebound from the surfaces many times as the air carrying them passes through the conductive airways to the regions where the O_2-CO_2 exchange takes place, with the probability of removal of contaminant gases along the conductive airways being dependent on their

solubility and reactivity with the mucous lining. Highly soluble gases such as sulfur dioxide are removed rapidly in the larger airways, whereas slightly soluble gases (*e.g.*, NO_2) reach the deep lung in relatively undiminished concentration.

All of these basic considerations apply to single contaminants. Where mixed contaminants are in the inhaled air, they may interact, and the resulting mixture may behave differently than either alone. As demonstrated by Amdur,[2] a mixture of SO_2 gas and a hygroscopic aerosol can produce much more respiratory tract irritation than either alone.

The properties of the inhaled particles, while essential to the determination of deposition site, are not sufficient in themselves. They must be considered in relation to the airway dimensions and branching angles of the species, and to its normal respiratory characteristics during the exposure. It should be recognized that these characteristics may change during the course of exposure if, for instance, the exposure causes bronchoconstriction, edema, excessive mucous secretions, or tissue damage. The resulting changes in effective airway dimensions or flow patterns could change the pattern of subsequent particle deposition.

In man, particles with aerodynamic diameters larger than a few microns deposit in the head and larger airways by impaction. As the inspired air spreads into smaller conductive airways the velocity rapidly diminishes, and beyond about the fifth airway generation the impaction probability becomes very low. At about the tenth generation of branching airway, the velocity becomes sufficiently low and the airway diameter sufficiently small for deposition by sedimentation to become significant for particles larger than about 1 μ. For submicron particles, Brownian motion deposition increases with decreasing particle size. Thus, the deposition probability in any given airway is dependent on the aerodynamic properties of the particle, on the airway dimensions, and on the velocity and transit time of the carrier air. Unfortunately, the airway dimensions and flow patterns are not well characterized in laboratory animals or in man, and predictive calculations on regional deposition usually have limited precision.

As previously discussed, the deposition mechanisms determine in large measure the deposition pattern within a given airway generation. The fate of a deposited particle is determined by the site of deposition. Insoluble particles impacting in the anterior unciliated nares are removed mechanically at periodic intervals by nose blowing and wiping, etc., while those impacting in the larynx and pharynx are swallowed rapidly. The particles deposited on the ciliated areas of the nose and on the tracheobronchial tree are transported on the moving mucous sheath to the larynx, and are swallowed within 1 day after deposition. "Insoluble" particles deposited in the nonciliated alveolar region are mobilized more slowly and by other mechanisms. They may dissolve and slowly enter the bloodstream, or they may be engulfed by phagocytes and be transported to the ciliary escalator or into the lymphatic system. The time scale for these mechanisms of elimination is quite variable, and depends on the physical and

chemical or surface properties of the particles. The particles which are mobilized and brought up on the mucociliary sheath and swallowed may cause injury to the gastrointestinal tract or may be dissolved therein and undergo systemic absorption.

Considering all the possible deposition probabilities in the various regions of the respiratory tract, the various time scales on which particles are mobilized, and the various storage reservoirs for materials systemically absorbed, it is generally advisable to perform experimental studies on the particular material of interest in order to properly assay its toxic and/or carcinogenic potential.

The discussion to follow will describe equipment and outline techniques developed for controlled inhalation exposures. Some aspects of contaminant atmosphere generation, delivery, and monitoring will be discussed in detail. For others, reference will be made to the discussions of Drs. Raabe, Hoffmann, and Bryan in Session I. Techniques used for experimental studies on regional particle deposition and bronchial clearance will also be discussed.

SYSTEMS FOR INHALATION EXPOSURE

Delivery Systems

Experimental inhalation studies require a reliable system for the delivery of known concentrations of contaminant to the test animals. In studies where the anticipated incidence of toxic symptoms among the test population will be low, large numbers of animals will be required. The techniques used must be capable of delivering to each animal a test atmosphere that remains reasonably constant throughout the daily exposure interval and from one exposure to the next. When the animals are exposed in chambers, it is important that the concentration variability from location to location within the chamber be kept as small as possible. A comprehensive review of the use of exposure chambers for research in animal inhalation has been given by Fraser *et al.*[3]

A potential complication in many total enclosure inhalation chamber studies is that the animals may absorb systemically material that was not inhaled. This can take place through direct skin absorption, but more often results from ingestion of contaminant deposited on the skin or fur of the exposed animal or its cage mates. There are several ways to minimize this problem. Direct inhalation exposure without surface deposition can be achieved by use of individual nasal tubes or face masks on each exposed animal. This approach has been used for particle clearance and metabolism studies on small numbers of test animals. At New York University Medical Center we have used nasal catheters to expose donkeys to SO_2 and cigarette smoke,[4] and have used face masks to expose them to ferric oxide particles (Fig. 1). The use of face masks, however, is usually not practical for studies on large populations because of the elaborate apparatus needed for restraining the animals' heads. The tubes and/or the restraint may alter the lung deposition pattern and physiologic response.

Fig 1 – A donkey is shown inhaling radioisotope-labeled ferric oxide particles from a spinning-disc generator output that passes through lower portion of form-fitting face mask The bronchial clearance of the deposited particles is determined from serial gamma measurements made with the scintillation detectors that are recessed into the lead brick shields on each side of the donkey

An alternative approach is the head exposure chamber. Here the nose or whole head of the animal passes through a minimal opening into an exposure chamber. Most of the animal is outside of the test atmosphere and it cannot ingest deposited particles. This technique is capable of providing a relatively uniform exposure to large numbers of small animals. However, the animals are usually confined in cylindrical or rectangular tubes not much larger than their bodies, and they require sedation for long periods of exposure.

Most inhalation toxicity studies are performed within total enclosure chambers whose size is determined by the number and size of the animals housed. Range-finding toxicity data needed to design the larger scale long-term studies can be performed on a limited number of animals in chambers as simple as a large battery jar. Intermediate range-finding data can often be obtained in small-scale exposure units of ~0.5 m^3 capacity for daily exposures for perhaps 3 to 6 months.

Most large general purpose inhalation chambers accommodate from 1 to 6 m^3 of animal cages and have permanently installed systems for air-conditioning, plumbing, air supply, and exhaust. They must be leak-tight, rigid, corrosion resistant, and capable of providing uniform temperature, humidity, and contaminant concentrations under a variety of operating conditions with minimal maintenance.

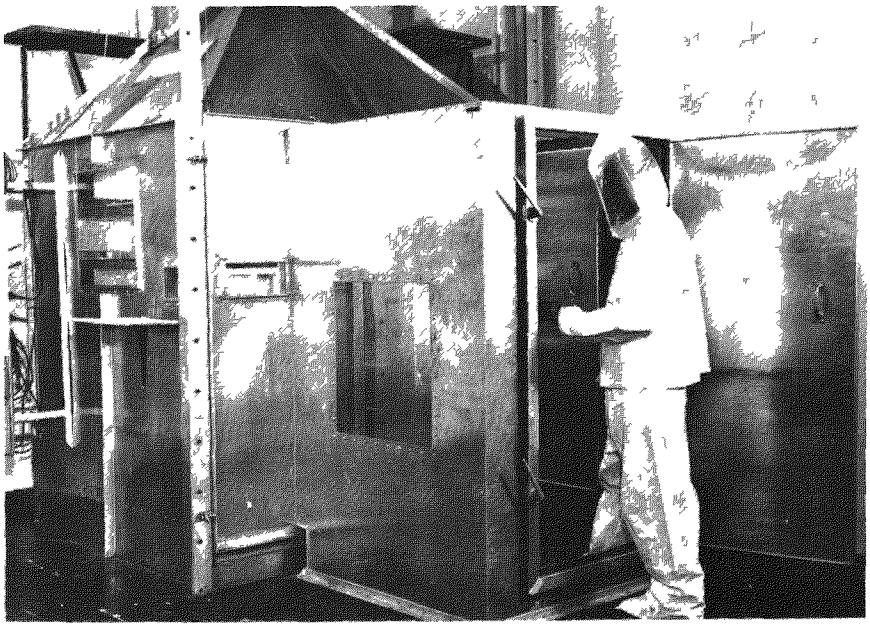

Fig. 2 – U.S. Public Health Service chamber for exposure to highly toxic test atmospheres. An attendant in protective clothing is shown entering the airlock.

Studies of cancer incidence resulting from exposure to airborne contaminants generally require life-long exposures of large populations exposed either daily or continuously in large exposure chambers General purpose chambers may be inadequate in terms of protection of the test animals against competing hazards, and for the protection of the animal handlers who could be exposed to carcinogens during transfer of the animals to separate housing space, or during cage and chamber maintenance between exposure intervals An exposure system specifically designed for inhalation studies with carcinogens will be described by Laskin in Session III [see p 338, these *Proceedings*] It consists of an interconnected series of sealed glove box chambers in which the animals remain under carefully controlled conditions from the time of their introduction into the colony as adolescent animals until they die or are killed All animal handling and equipment manipulation within the chambers is done from the outside with long rubber gloves Another approach is shown in Fig 2, which illustrates a walk-in type chamber at the Public Health Service's Bureau of Occupational Health and Safety Laboratory in Cincinnati The animals remain in the main body of the chamber for the duration of the study, and are exposed to a beryllium aerosol for 6 hr per day Animal feeding and cleanup operations are performed after an exposure by an attendant who wears disposable protective clothing and respiratory protection, and who enters and leaves the chamber through an air lock

Elements of Chamber Design

Large-scale multipurpose animal inhalation chambers should be capable of maintaining uniform concentrations of particles in air, since it is much harder to produce uniform particle distributions than uniform gas or vapor concentrations Chambers for cigarette smoke exposure, where the concentration is high enough for rapid particle agglommeration, present special problems, these will be discussed in detail by Hoffmann in Session I [see p 182, these *Proceedings*] The remainder of this discussion will be concerned with chambers for the lower aerosol concentrations used in most inhalation toxicology studies

Uniform aerosol distributions can most easily be maintained in a chamber where the inlet air is fed in at the top and withdrawn at or near the bottom The inlet and outlet of the chamber must be designed so that the concentration of air contaminant across a horizontal cross section of the chamber is uniform For practical purposes, this kind of uniformity can be obtained only with a cross section that is a regular polygon or circular Although some chambers are designed with circular, hexagonal, or octagonal cross sections, most use a square cross section One important reason is the ease of fabrication, another is that from the standpoint of operation, loading and unloading of cages is easier, especially since a large access door can readily be accommodated with a square cross section In long term studies, the effects of spatial variations in contaminant concentration within the chamber can be compensated for by the systematic rotation of the cage locations within the chamber during reloading

Most modern chambers utilize a pyramidal top to provide the dispersion space needed for the creation of uniform flow patterns The chamber exhaust is drawn through a distributing plenum below the exposure zone The plenum is generally designed so that the exhaust air can readily be removed without entrained liquid and/or solid waste from the animals above

From an operational point of view, one major factor in chamber design is the ease with which the chambers can be kept clean In many laboratories cleaning is performed with a high-pressure water hose after each animal occupancy The chambers in these laboratories are designed with pitched floors, center drains, and smooth interior walls which are easily hosed down For routine types of exposures the drain from each chamber can be run into a sanitary sewer line When highly toxic materials such as potential carcinogens are handled, however, the liquid waste should be collected in a sealed container for special handling

The materials of construction used for animal inhalation chambers are determined by the nature of the contaminants to be introduced, and to some extent by the preference of the chamber operator The important characteristics are the quality and shape of the interior surfaces and the ease with which they can be kept clean The surfaces should be smooth and nonabsorbent, or at least lined with a nonabsorbent material Modern chambers are generally designed with walls of aluminum alloy or stainless steel and windows of glass or lucite Chambers are sometimes constructed in clusters with common walls rather than as separate units Five 1 6 m^3 general purpose inhalation chambers at New York University Institute of Environmental Medicine were built in a row The University of Rochester has built a cluster of hexagonally shaped chambers

The number and size of exposure chambers needed for a study depends on the animal volume to be housed To obtain uniform aerosol exposures for sedentary animals in cages, the animal volume should be limited to 5% of the chamber volume Five percent is a practical limit for animals housed in individual or compartmentalized cages that completely fill a chamber [3] Also, because of the metabolic heat load, it is difficult to maintain the air temperature in chambers with greater animal loadings

Test Animals

The choice of animal species for inhalation studies depends on many factors and is beyond the scope of this discussion Animals ranging in size from mice to donkeys have been used, and generally have been housed in individual cages or in small groups In this sense, the animals are exposed in an abnormal physical and social environment, and their response may differ from that of similar animals exposed to the same levels of toxicant in their natural environment Palmes and Del Pup[5] are investigating the use of mouse populations as test objects for evaluation of toxic effects of environmental agents Each population consists of 400 cross-bred mice living in a common cage, with the population size maintained by the addition of randomly selected weanlings to replace dying animals

GENERATION OF TEST ATMOSPHERES

Aerosol Generation Techniques

The generation of a test aerosol having the desired combination of physical and chemical properties for an inhalation study is one of the more formidible tasks in experimental toxicology There are relatively few commercially available aerosol generators capable of producing a stable reproducible aerosol over extended intervals Those that are available and reliable may not be capable of producing the desired airborne concentration, particle size distribution, shape, density, charge, surface properties, etc. Furthermore, it is very difficult to obtain useful data on the characteristics of commercial aerosol generators and on the manner in which their output is affected by operational variables The vendors, who probably designed their devices for other applications, usually do not have useful data to provide For example, the common drugstore variety of DeVilbiss No 40 nebulizer has been widely used as a laboratory aerosol generator for many years, but its performance under varying operating conditions has only recently been described in detail [6] The performance characteristics of this and several other aerosol generators will be discussed in Session I by Raabe [see pp 124–142, these *Proceedings*]

Comprehensive reviews of the types of aerosol generators which are available or have been described in the literature, albeit with less information on their characteristics, have been made by Fraser *et al*,[3] Silverman,[7] and Lodge [8] A review of techniques and equipment for producing monodisperse aerosols has been prepared by Fuchs and Sutugin [9]

Aerosol generators can be divided into two types those that produce condensation aerosols, and those that produce dispersion aerosols In the former type, the material to be aerosolized is dispersed in the vapor phase and allowed to condense on airborne nuclei As demonstrated by LaMer and Sinclair,[10] a monodisperse aerosol can be produced if the condensation takes place under carefully controlled conditions The particles will be liquid and spherical, unless the material vaporized has a melting point above ambient temperature In this case, the particles will solidify, and if crystalline may form nonspherical shapes

Improvements in the basic LaMer-Sinclair design have been described by Muir [11] Rapaport and Weinstock[12] have described a condensation aerosol generator that is simpler, less expensive to produce, and requires less critical control of temperature and flow rate for the production of monodisperse aerosol A more sophisticated version of this generator has been described by Lui *et al* [13] This type of generator is capable of producing high quality aerosols of high temperature boiling, low vapor pressure liquids – such as dioctyl phthalate, triphenylphosphate, and sulfuric acid – in the size range of about 0 05 to 1 μ An apparatus for producing monodisperse condensation aerosols of lead, zinc, cadmium, and antimony using a high frequency induction furnace has been described by Homma [14]

A summary of techniques for producing radioactively labeled monodisperse condensation aerosols with 18 organic compounds and 8 inorganic materials has been presented by Spurny and Lodge.[15]

Dispersion aerosol generators may be further subdivided into two classes – wet and dry. Dry generators comminute a bulk solid or packed powder by mechanical means, usually with the aid of an air jet. They often include an impaction plate at the outlet for removal of oversize particles and for breaking up aggregates. The aerosol particles produced are typically composed of solid, irregularly shaped particles which have a broad range of sizes. Also, the rate of generation is usually not perfectly uniform, since it depends on the uniformity of hardness, or friability, of the bulk material being subdivided, as well as on the uniformity of the feed-drive mechanism and air-jet pressure.

The characteristics of a variety of different types of dry dust generators have been described by Ebens,[16] including the widely used Wright Dust Feed.[17] Among the more difficult kinds of dry dust aerosols to generate are plastics that develop high electrostatic charges. Laskin *et al.*[18] have described two types of generator for such materials. One uses a high-speed fan to create a stable fluidized bed from which aerosol can be drawn; the second uses a high-speed grinder to comminute a block of solid material.

Other generator designs developed for "problem" dusts include those by Dimmock[19] for viable dusts, by Brown *et al.*[20] for deliquescent dust, and by Timbrell *et al.*[21] and Holt and Young[22] for fibrous dust.

Wet-dispersion generators break up bulk liquid into droplets. If the liquid is nonvolatile, the resulting aerosol will be a mist or fog. If a volatile liquid is aerosolized, the resulting particles will be composed of the nonvolatile residues in the feed liquid, and will be much smaller than the droplets dispersed from the generator. Solid particles can be produced by nebulizing salt or dye solutions or particle suspensions. If aqueous solutions are used, the particles will, of course, be water soluble, and may be hygroscopic – important factors to consider if these aerosols are to be inhaled by test animals.

Solid insoluble aerosols can be produced by nebulizing particle suspensions. One technique is to prepare a suspension of the particles in which the size distribution is sufficiently dilute in the liquid phase so that the probability of more than one particle being present in each droplet is acceptably small. This will result in a high vapor-to-particle ratio, thus limiting the mass concentration of the aerosol produced. Another approach is to use a colloid as the feed liquid. In this case, the diameter of the colloid particles can be orders of magnitude smaller than the particles in the resulting aerosol. Thus, the volume of the colloid particles in each droplet is proportional to the volume of the droplet, and the size of the dried aggregate particles is determined by the solids content of the sol.

Solid aerosols resulting from droplet evaporation will generally be spherical, but not always. Too rapid solvent evaporation, low pH, and the presence of impurities may cause the dried particles to be wrinkled or to assume various shapes.

A variety of techniques can be used to subdivide bulk liquid into airborne droplets. In most cases the liquid is accelerated by the application of mechanical, pneumatic, or centrifugal forces and drawn into filaments or films which break up into droplets because of surface tension. Centrifugal pressure nozzles and fan spray nozzles use hydraulic pressure to form a sheet of liquid which breaks up into droplets, but these generally have high liquid throughout and are rarely used for inhalation studies.

A commonly used type of aerosol generator is the two-fluid nozzle, which uses pneumatic energy to break up the liquid. Several laboratory-scale compressed-air-driven nebulizers have been described in detail by Mercer *et al.*[6] and will be discussed by Raabe in the next session. Some of these are made of glass, which not only makes them fragile but also limits their precision of manufacture and reproducibility. Ready reproducibility led Whitby to select the British Collison[23] nebulizer for his atomizer-impactor aerosol generator.[24] Other commercially available nebulizers, including those of Wright[25] and Dautrebande,[26] are machined to close tolerance from plastic materials. All of these two-fluid atomizers produce polydisperse aerosols, although relatively narrow size dispersions can be obtained with Whitby's atomizer-impactor[24] and Dautrebande's D-30.[26]

Rotary atomizers, such as the spinning disc, utilize centrifugal force to break up the liquid, which undergoes an acceleration as it spreads from the center to the edge of the disc. The liquid leaves the edge of the disc as individual droplets or as ligaments which disintegrate into droplets. When operated with low liquid feed rates and high peripheral speeds, these atomizers can produce monodisperse aerosols. A spinning disc generator designed specifically for the production of test aerosol for inhalation studies of hazardous materials has been described by Lippmann and Albert.[27]

Monodisperse test aerosols can also be produced by a variety of techniques that break up a laminar liquid jet into uniform droplets. Most of them vibrate a capillary at high speed with a variety of transducers and types of motion. Dimmock's[28] generator, for example, uses transverse vibrations, while Ström's[29] uses axial vibrations. Wolf[30] uses a vibrating reed, wetted to a constant length by passage through a liquid reservoir, to create the droplet stream.

Electrostatic atomization can also produce monodisperse aerosols. Electric charges on a liquid surface act to decrease the surface tension. Liquid flowing through a capillary at high voltage is drawn into a narrow thread that breaks up into very small droplets.[31,32]

Commerical ultrasonic aerosol generators are available which vibrate a liquid surface at high frequency, resulting in the disintegration of the surface liquid into a polydisperse droplet aerosol. For mass median droplet diameters below 5 μ the transducer must vibrate at a frequency greater than 1 megacycle.[9]

Production of Gas and Vapor Test Atmospheres

The selection of a technique for the production of a uniform gas mixture for dynamic exposure systems should be based on consideration of many factors These include the concentration range and uniformity required, the potential for losses to and/or contamination from the surfaces in the gas generator or connecting lines, the amount of time over which the generator must operate, the cost of operation, the downtime needed for recharge and/or maintenance, etc. Since these considerations will be discussed by Bryan in Session I [see p 193, these *Proceedings*], they will not be discussed further here. Other general discussions on the generation of gas and vapor atmospheres have been prepared by Silverman[7] and Lodge [8] Hersh[33] has prepared a detailed discussion of some less well-known techniques (*e g*, liquid piston feeds, microflow-through channels, diffusion across channels and barriers, stream splatters for attenuation, and methods based on evaporation, electrolysis, chemical conversion, and irradiation.

MONITORING TEST ATMOSPHERES

Characterization of test atmospheres in inhalation chambers is an essential aspect of the hazard evaluation and deserves careful consideration. Special attention should be given to time and spatial variation within the chamber, sample losses and/or alterations between the probe inlet and analytical instruments, and the accuracy, specificity, and precision of the analyses Further discussion of these important topics will be presented in Session I by Raabe for aerosols and Bryan for gases and vapors.

STUDIES ON REGIONAL DEPOSITION AND CLEARANCE

Studies of regional particle deposition have been performed by a variety of techniques. Sacrifice after inhalation exposure can be performed on numerous small animal species, and large numbers of animals can be used. The various regional depositions can be determined by chemical or radiometric assay. The distribution of the particles and patterns of motion of the deposited particles on the larger airways can be evaluated by microscopic examination of the airway surfaces However, interpretation of these data is complicated by the possible alteration of the airway surfaces and their deposits between the death of the animal and the microscopic assay. Complications may arise from processes associated with death and with the fixation of the tissue for microscopic examination

Studies of regional particle deposition in man have been performed by other techniques One approach is based on the careful analysis of the exhaled aerosol after controlled inhalations, a second technique employs radioactive test aerosols and is based on the measurement of the deposited aerosol.

Lindahl *et al*,[34,35] Brown *et al*,[36] and Altshuler *et al*[37] have used the former technique, in which variations in the concentration of the exhaled

aerosol are associated with the region from which the expired aerosol was presumed to have originated. The validity of these data depends upon the accuracy of the association between the various exhaled air fractions and their presumed sources in the lung depths. The accuracy is also influenced by the assumptions made about respiratory dead space volume and the amount of mixing that takes place between the tidal air and the residual air in each breath.[37]

The second approach is to use radioactive test aerosols and measure the activity in both the exhaled and deposited fractions. Dr. Roy E. Albert and I[38] have been using this technique in our studies of regional aerosol deposition in man. Measurements made immediately after the end of an inhalation of labeled monodisperse aerosol indicate the fractions deposited in the head, trachea and whole lung. The exhaled aerosol, collected on a respirator canister, is also measured. A retention measurement made upon completion of bronchial clearance indicates the alveolar deposition. It has been demonstrated that the cleared material is bronchial clearance because of its passage through the trachea and esophagus during clearance, and its subsequent appearance in the stomach and gastrointestinal tract. With this technique, it is possible to subdivide the inhaled aerosol into an exhaled fraction and deposition within the following functional regions: anterior unciliated nares, ciliated nasal passages and nasopharynx, larynx, trachea, tracheobronchial tree, and alveolar.

The accuracy of these data is dependent on several assumptions. One is that all of the particles deposited on the ciliated airways are cleared within the bronchial phase, and that none of the alveolar deposit is mobilized via the bronchial tree during this time interval. The justifications for these assumptions are twofold: (1) the very sharp discontinuity between the bronchial clearance phase, which lasts anywhere from 4 to 24 hr in man, and (2) the subsequent slower particle clearance by other mechanisms. Also, many studies with small animals have shown the bronchial tree to be clear of particles within 1 day after inhalation.

Regional deposition can be determined from an initial and a 24-hr retention measurement. Studies on the nature of the bronchial clearance process require additional measurements. In practice, most of the inhalation studies with radioactive aerosols just described involved serial measurements of retained activity throughout the 1st postexposure day. The data thus obtained enable us to define normal patterns of bronchial clearance in man and to describe several abnormal bronchial clearance patterns seen in heavy cigarette smokers and patients with lung disease.[39] These include: (1) an extended delay in the onset of clearance or between clearance phases, (2) a spasmodic type of clearance with intermittent tracheal blockage, and (3) an extended period of clearance arrest with a retrograde movement of particles from the hilar region to more distal lower lung regions. More recently, we have found that most bronchitic patients and a sizable minority of cigarette smokers had higher tracheobronchial deposition than nonsmoking individuals.

Inhalation studies with monodisperse test aerosols can provide needed information on pulmonary dimensions and function. For example, intersubject differences in tracheobronchial deposition in the studies previously described can be associated with variations in cross-sectional area in the conductive airways. Individuals with chronic bronchitis and asthma have much higher tracheobronchial depositions for a given particle size than do normal subjects, and the observed differences may provide a basis for the development of a diagnostic clinical test.

Several other aspects of pulmonary function in man have been investigated with particles of about 0.5 μ diameter unit density.[40] Total respiratory tract deposition is minimal at this size. For larger particles, inertial and gravitational forces result in increased deposition, while for smaller particles increased Brownian motion displacement results in greater deposition. Thus, 0.5-μ particles serve as tracers of airflow and, as demonstrated by Altshuler[41] and Muir,[42] can be used to assess convective mixing in the lung.

Submicron monodisperse aerosols can also be used to measure the average alveolar dimension in life. In tests performed by Palmes *et al.*[43] the subject inhales a volume that is relatively large compared to his respiratory dead space volume. Thus, most of the aerosol is contained in air spaces of relatively constant dimension. The decrease in aerosol concentration during breath-holding in such airways is therefore a function of an average dimension of these airways. Aerosol deposition can be measured by having the subject exhale twice the volume he inhaled. This larger volume contains virtually all of the inhaled particles which did not deposit. Some aerosol is lost on inspiration and expiration as it passes through the conductive airways. But this loss is independent of duration and breath-holding, and the incremental loss with increasing duration of breath-holding represents the deposition under constant volume conditions. Experiments were conducted with both 0.15-μ particles, for which displacement is primarily by diffusion, and 0.75-μ particles, for which gravitational sedimentation is predominant. For both sizes, the recovery of aerosol decreased exponentially with time of breath-holding up to about 30 sec.

These findings are inconsistent with simple spatial models for the alveolar region utilized in previous lung models, and a new alveolar spatial unit has been proposed by Altshuler.[40] It consists of an axial duct surrounded by open-faced compartments and would have a parabolical velocity distribution in the duct part with positive boundary velocity at the open boundaries with the surrounding alveolar sac compartments.

Particle clearance dynamics and pathways have been studied with several techniques. One is the serial sacrifice of exposed animals at suitable time intervals after the test inhalation, with examination of the distribution of retained particles on the surfaces of the respiratory tract. A second approach is to make external measurements of radioactive, gamma-emitting isotopes incorporated in the particles, as previously discussed. This technique has the advantage of minimal interference with normal biological processes, and

produces no change in the test animal, who can be the subject of repeated studies. It is most useful for studies of bronchial clearance where the particles are removed to the gastrointestinal tract within the 1st day. It cannot be used effectively for studies of longer-term particle clearance, where the particles may be mobilized to physically adjacent areas such as the lymph nodes, unless additional studies on sacrificed animals are made to determine the magnitude of such translocation.

The effect of respiratory irritants on bronchial clearance has been studied with donkeys used as test animals.[4,44] In donkeys, as in man, the amount and pattern of bronchial clearance in the same individual is reproducible from test to test if the aerosol and breathing parameters are the same. Thus, the normal clearance pattern for a given aerosol in a given animal can serve as a basis for determining the effect of irritants in producing alterations in normal clearance patterns. Each animal must be individually calibrated, since variations among donkeys, and among men, are considerable from one to the next. We have produced substantial retardations in bronchial clearance in donkeys, using both sulfur dioxide and whole fresh cigarette smoke.

Spritzer and Watson[45] have described a technique for the complete collection of the bronchial mucus in rats. It permits the direct collection of particles cleared from the lung by mucociliary action. A polyethylene tube is inserted into the lower end of the esophagus and brought out through the left side of the rat, where it drains into a 100 cm^3 polyethylene bottle. The animals can be maintained for up to 7 days after surgery by subcutaneous injections of glucose and saline twice daily. Using this collection technique, Watson *et al.*[46] reported that mucociliary clearance kinetics after inhalation and intratracheal injection were similar.

Direct visual examination of particle movement on the smaller ciliated airways of mammalian lungs is not possible. However, Kilburn[47] has been able to observe the motion of insoluble particles in the lungs of bullfrogs by turning the lungs inside out through the glottis, enabling the living lung to be examined microscopically without surgery. In these simple lungs, the muscular ridges are analogous to the conductive airways of more complex lungs. Kilburn reported that the mucous velocity on the major ridge was 2.2 cm/min, and decreased by half on each generation of annular and smaller ridge.

SUMMARY

Basic considerations, equipment, and procedures have been reviewed for two categories of experimental inhalation studies: (1) those on the regional deposition and clearance dynamics of inhaled contaminants, and (2) those on the dose-response relationships for specific contaminants. Knowledge gained in the first type of study is essential to the proper design and interpretation of data from the second type, since the relation between the airborne concentration inhaled and the tissue dose at the site of toxic action depends on the distribution of the deposited material and the residence time on the respiratory epithelium.

Basic considerations involved in the design and selection of air contaminant generation and of delivery and monitoring systems were reviewed. Techniques for aerosol generation were described that permit the production of aerosols with the desired physical and chemical characteristics and size dispersion. Also described were a variety of delivery systems – including nasal tubes and face masks, head exposure chambers, and total enclosure chambers. Techniques for gas and vapor generation and the monitoring of test atmospheres were not described in detail, since they are covered in these Proceedings by Bryan and Raabe. Experimental studies of aerosol deposition in and clearance from the respiratory tract by a variety of techniques were summarized.

REFERENCES

1. T. T. Mercer, Aerosol production and characterization. *Health Phys.*, 10 (1964) 873–887.
2. M. O. Amdur, The effect of aerosols on the response to irritant gases. In, *Inhaled Particles and Vapours*, Vol. I, pp. 281–294, C. N. Davies (ed.). Pergamon Press, London, 1961.
3. D. A. Fraser, R. E. Bales, M. Lippmann, and H. E. Stokinger, *Exposure Chambers for Research in Animal Inhalation,* Public Health Monograph No. 57, P.H.S. Publication No. 662, USGPO. Washington, D.C., 1959.
4. J. R. Spiegelman, G. D. Hanson, A. Lazarus, R. T. Bennett, M. Lippmann, and R. E. Albert, Effect of acute sulfur dioxide exposure on bronchial clearance in the donkey. *Arch. Environ. Health,* 17 (1968) 321–326.
5. E. D. Palmes and J. Del Pup, Mouse populations as test animals – A progress report. *Am. Ind. Hyg. Assoc. J.*, in press.
6. T. T. Mercer, M. I. Tillery, and H. Y. Chow, Operating characteristics of some compressed air nebulizers. *Am. Ind. Hyg. Assoc. J.*, 29 (1968) 66–78.
7. L. Silverman, Experimental test methods. In, *Air Pollution Handbook,* pp. 12:1–12:48, P. L. Magill, F. R. Holden, and C. Ackley (eds.). McGraw-Hill, New York, 1956.
8. J. P. Lodge, Production of controlled test atmospheres. In, *Air Pollution,* 2nd edition, Vol. II, Chap. 27, A. C. Stern (ed.). Academic Press, New York, 1968.
9. N. A. Fuchs and A. G. Sutugin, Generation and use of monodisperse Aerosols. In, *Aerosol Science,* pp. 1–30, C. N. Davies (ed.). Academic Press, London, 1966.
10. V. K. LaMer, and D. Sinclair, An Improved Homogeneous Aerosol Generator, OSRD Report No. 1668. U.S. Dept. of Commerce, Washington, D.C., 1943.
11. D. C. F. Muir, The production of monodisperse aerosols by a LaMer-Sinclair generator. *Ann. Occup. Hyg.*, 8 (1965) 233–238.
12. E. Rapaport and S. G. Weinstock, A generator for homogeneous aerosols. *Experimentia,* XI/9 (1955) 363.
13. B. Y. H. Liu, K. T. Whitby, and H. S. Yu, A condensation aerosol generator for producing monodispersed aerosols in the size range 0.036 μ to 1.3 μ. *Jour. de Reserches Atmospheriques* (Paris, France), 3 (1966).
14. K. Homma, Experimental study for preparing metal fumes. *Ind. Health,* 4 (1966) 129–137.
15. K. R. Spurny and J. P. Lodge, Jr., Radioactively labeled aerosols. *Atmos. Environ.*, 2 (1968) 429–440.
16. R. Ebens, Erfahrungen mit einigen kontinuierlich arbeitenden Staubdosiervorrichtungen zur Aufrechterhaltung konstanter Staubkonzentration in Prüfanlangen. *Staub,* 29 (1969) 89–92.

17 B M Wright, A new dust feed mechanism *J Sci Inst*, 27 (1950) 12–15
18 S Laskin, S Posner, and R Drew, New designs for dust generating systems for inhalation studies Presented at 1968 AIHA Annual Meeting, St Louis, Mo, May 1968
19 R L Dimmock, Jet disperser for compacted powders in the 1–10 μ range *A M A Arch Ind Health*, 20 (July 1959) 8–14
20 J R Brown, J Horwood, and E Mastromatteo, A dust dispenser for deliquescent material *Ann Occup Hyg* 5 (1962) 145–147
21 V Timbrell, A W Hyett, and J W Skidmore, A simple dispenser for generating dust clouds from standard reference samples of asbestos *Ann Occup Hyg* 11 (1968) 273–281
22 P F Holt, and D K Young, A dust feed mechanism suitable for fibrous dust *Ann Occup Hyg* 2 (1960) 249–256
23 Methylene Blue Particulate Test for Respirator Canisters, British Standards Institute No 2577 2 Park St, London W 1, 1955
24 K T Whitby, D A Lundgren, and C M Peterson, Homogeneous aerosol generators *Intern J Air Water Pollut* 9 (1965) 263–277
25 B M Wright, A new nebulizer *Lancet*, (1958) 24–25
26 L Dautrebande, *Microaerosols* Academic Press, New York, 1962
27 M Lippmann and R E Albert, A compact electric-motor driven spinning disc aerosol generator *Am. Ind Hyg Assoc J* 28 (1967) 501–506
28 N A Dimmock, Production of uniform droplets *Nature* 166 (1950) 686–687
29 L Strom, The generation of monodisperse aerosols by means of a disintegrated jet of liquid *Rev Sci Inst* 40 (1969) 778–782
30 W R Wolf, Study of the vibrating reed in the production of small droplets and solid particles of uniform size *Rev Sci Inst*, 32 (1961) 1124–1129
31 M A Nawab, and S G Mason, The preparation of uniform emulsions by electrical dispersion *J Coll Sci* 12 (1958) 179–187
32 E P Yurkstas, and C J Meisenzehl, Solid Homogeneous Aerosol Production by Electrical Atomization, University of Rochester Atomic Energy Report, UR 652, Oct 30, 1964
33 P A Hersch, Controlled addition of experimental pollutants to air *J Air Pollut Contr Assoc* 19 (March 1969) 164–172
34 H D Landahl, T N Tracewell, and W H Lassen, On the retention of airborne particulates in the human lung, II *A M A Arch Ind Hyg Occup Med* 3 (1951) 359
35 H D Landahl, T N Tracewell, and W H Lassen, Retention of airborne particulates in the human lung, III *A M A Arch Ind Hyg Occup Med* 6 (1952) 508
36 J H Brown, K M Cook, F G Ney, and T Hatch, Influence of particle size upon the retention of particulate matter in the human lung *Am J Public Health*, 40 (1950) 450
37 B Altshuler, E D Palmes, and N Nelson, Regional aerosol deposition in the human respiratory tract In, *Inhaled Particles and Vapours II* pp 323–337, C N Davies (ed) Pergamon Press, Oxford, 1967
38 M Lippmann, and R E Albert, The effect of particle size on the regional deposition of inhaled aerosols in the human respiratory tract *Am Ind Hyg Assoc* 30 (1969) 257–275
39 R E Albert, M Lippmann, and W Briscoe, The characteristics of bronchial clearance in humans and the effects of cigarette smoking *Arch Environ Health*, 18 (1969) 738–755
40 B Altshuler, Behavior of airborne particles in the respiratory tract In, *Ciba Foundation Symposium on Circulatory and Respiratory Mass Transport*, pp 215–231, G E W Wolstenholme and J Knight (eds) J & A Churchill Ltd, London, 1969

41 B Altshuler, The role of the mixing of intrapulmonary gas flow in the deposition of aerosol In, *Inhaled Particles and Vapours* pp 47–53, C N Davies (ed) Pergamon Press, London, 1961
42 D C F Muir, Distribution of aerosol particles in exhaled air *J Appl Physiol* 23 (1967) 210–214
43 E D Palmes, B Altshuler, and N Nelson, Deposition of aerosols in the human respiratory tract during breath holding In, *Inhaled Particles and Vapours II* pp 339–349, C N Davies (ed) Pergamon Press, London, 1967
44 R E Albert, J R Spiegelman, S Shatsky, and M Lippmann, The effect of acute exposure to cigarette smoke on bronchial clearance in the miniature donkey *Arch Environ Health,* 18 (1969) 30 41
45 A A Spritzer and J A Watson, The measurement of ciliary clearance in the lungs of rats *Health Phys* , 10 (1964) 1093 1097
46 J A Watson, A A Spritzer, J A Auld, and M A Guetthoff, Deposition and clearance following inhalation and intratracheal injection of particles *Arch Environ Health,* 19 (1969) 51–58
47 K H Kilburn, Mucociliary clearance from bullfrog lung *J Appl Physiol* 23 (1967) 804–810

DISCUSSION

A. P. Wehner: I feel that a parameter often neglected in the definition of environment is the percentage of respirable fraction in a given aerosol concentration

M. Lippmann: Would you define *respirable?*

Wehner: I mean particles of the size that, according to our present knowledge, should reach the subject's respiratory tract or alveoli We have some fairly good ideas that particles beyond a given unit density size will not enter the respiratory tract or the alveoli

Lippmann: There are many cut-off points There are particles which are too big to enter the nose (*i e* , they can't be aspirated), and those which do not enter the alveoli because they are removed efficiently in the conductive airways

Wehner: With a given concentration of an aerosol, it should be stated what fraction of this aerosol is actually of respirable size

Lippmann: I couldn't agree with you more. I would like to see *respirable* defined in each case, however It depends upon the properties of the material Some materials have their toxic action in the tracheobronchial tree, so the definition of what gets to the alveoli may not be important Whether or not a material is "respirable" depends on the tissue site of action for that particular material

S. Laskin: I have several comments In relation to the previous commentator's remarks, the problem of "respirable" size must be looked at in terms of careful characterization of the particle distribution that the animal is being exposed to, and this poses another problem What is getting into the animal?

This is a never never land that ranges from some of the tobacco studies in which it is questionable, because of the conditions of the experiment, whether any may be getting in, to those experiments involving massive exposures In the end we have to determine this analytically from animal tissue analysis, or by some other means of evaluation

I would like to mention an old experiment of ours with aldehydes some years ago We set up a known concentration under exact control in an empty chamber This procedure is the one generally used to test out an exposure Everything went beautifully However, the moment we put the animals in, the concentration dropped to zero It disturbed us, but within a very short time we realized what the problem was The aldehyde was all absorbed on the animals' fur, and nothing penetrated the lungs To emphasize this note of caution in inhalation experiments Be sure the stuff is getting in I'm sure other people would give the same warning

To go on to some other comments Dr Lippmann estimated the loading of a chamber as approximately 5% This is a maximum that can be reached under special conditions The decision as to how many animals you can put into a closed box relates to ventilation conditions, not really to the oxygen supply There's sufficient ventilation to meet oxygen or CO_2 requirements The question largely relates, then, to thermal loading Animals packed into a box emit a lot of calories It is not unusual to see a rise of 10 degrees in a chamber, even with a reasonable amount of air conditioning If you air condition from the entry of the system, you freeze your top layer of animals So you have to reach a balance, a compromise, of air turnover and space between the animals so that you don't have any significant temperature rise

Another factor, of course, is the humidity rise The air exhaled is saturated, and it's a common experience in small chambers to see the entire chamber suddenly fog up and reach 100% saturation Obviously this changes the nature of the contaminants Old studies, referred to by T Hatch and a number of other workers [see P Drinker and T Hatch, *Industrial Dust* 2nd ed McGraw-Hill, New York, 1954], illustrate that although humidity does not normally affect particle aglomeration, saturation conditions do You no longer have the same particle

The third item to consider is the behavior of the animals Most of us with a background in toxicology worry about controls, as Dr Saffiotti pointed out, the need is for very careful controls We tend to throw animals into a box, thinking "well, we've got control data on some animals back in the laboratory colony" But it doesn't quite work this way Animals respond to handling They behave quite differently And the hamster is notorious for this It loves to be handled It grows better, it does better, it lives longer if it is handled every day If you put it off in a corner and forget about it and just feed it and make sure you are giving it the best of food and sanitary conditions, it doesn't do as well

Lippmann: Thank you. I certainly didn't want to leave the impression that monitoring the chamber atmosphere isn't one of the most important things we have to do. Again, I was trying to avoid getting into what Dr. Bryan is going to discuss.

U. Saffiotti: A practical question, since we have this gathering of experts. I have been trying for some time to design a device for the direct administration of inhalants into the tracheobronchial tract by an intratracheal cannula, applicable to the small rodent. The purpose is to bypass the nasal cavities, which have been shown to exert a marked filtering effect.

We made a preliminary attempt a few years ago with an intratracheal cannula for hamsters that was connected to a microchamber where a dusty atmosphere was generated. Of course the problem was exhaled air, with humidity and clogging of the small tube. Do you know of any effort along these lines, or does anyone here have any suggestions on how to design such a gadget? I think if we had a way to introduce the test atmosphere directly into the trachea of rodents we could do a lot of inhalation experiments with exposure of the lower parts of the respiratory tract.

Lippmann: In our laboratory we have used nose tubes in donkeys, and we do this to avoid nasal absorption of the irritant.

Y. Alarie: We have worked a great deal on what Dr. Saffiotti just asked, and in small rodents it is practically impossible to do long-term chronic studies with face masks. About the longest time period is a 3- to 4-hr exposure, and when the animals get out of the face mask they usually have blood in their noses and other undesirable conditions – this happens particularly in rats.

One group of animals on which face masks have worked absolutely beautifully are the primates. With cynomolgus monkeys we seat the animal in a chair and affix a face mask to its head. They take it very nicely, and we have conducted experiments 6–8 hr a day, for up to 18 months, with this exposure method.

Also, I think that with the primate the face mask is much better and less expensive than the exposure chamber; the construction of these large chambers is quite costly. You would also need a penthouse with pumps and an air-conditioning system, and the whole paraphernalia of things that go with exposure chambers. With a chair and face mask it's very easy to control the exposure, and you can dose your animals individually. The amount of pollutants needed is also much reduced.

F. Homburger: We have been frustrated for more than 2 years in attempts to eliminate nose breathing in the mouse. We have finally succeeded by a very simple method. A small elastic rubber band is placed around the nose of the mouse, leaving the mouth open. This is accomplished by passing a conical metal tube over the tip of the nose of the mouse and then slipping a rubber band down over the wide end of the cone on the nose. Occlusion of the nose may be

ascertained by dipping the nose into water The nasal occlusion is well tolerated, with the following reservations there are strain differences, in mice, of the anatomical structure of the nasal pharynx Some strains will die from aerophagia within 2 to 6 hr after occlusion of the nose, with rupture of the intestine, other strains will tolerate the device for up to about 10 12 hr We have also tried the method on rats, with less success, and are currently using it on hamsters

We don't place the mice into our chambers any more, but have them living outside with a collar, the collar being made of felt on the inside and aluminum on the outside In this way, 10 mice can live in a cage and don't chew each others' collars The collars fit into openings in the outside well of the chamber so that only the head is exposed

Alarie: There is some very serious objection to occluding the noses of animals With monkeys as well as with mice, the respiratory patterns change very drastically If you are doing inhalation and deposition studies, you will find a lot of differences depending on whether your animals are breathing through the mouth with their noses obstructed or through the nose After all, mice, rats, and guinea pigs are strictly nose breathers Monkeys are primarily nose breathers, they will breathe through their mouths from time to time, but usually through the nose If you subject them to irritants such as cigarette smoke, they will switch from nose breathing to mouth breathing very frequently

Laskin On the problem of intubating a small animal, I think many people have tried and failed, but I think it is a question of working on techniques

However, there is a large amount of literature about people who have successfully put tracheal cannulas in dogs and have designed face masks for rabbits I believe Paul Morrow did some of the early studies It's quite nice in a dog, as you can literally put a valve in the neck, open it periodically, go in, and put a pipeline in With smaller animals you have a difficult problem just handling it – keeping the thing closed

With reference to head exposure, I would like to mention the early studies in the uranium monographs published at Rochester by Voegtlin and Hodge. They describe the development of a number of head exposure systems, covering a range of animals from rats up through dogs These systems were quite successful

Alarie: If I may make one final comment on exposure chambers We have talked about all kinds of things – ventilation and loading, and so forth – but there is one thing we have not talked about – the urine and feces of these animals that drop into the bottom of the chamber I visited several investigators in this country, and some of them were very proud of their generation systems and very proud to maintain 5 ppm of sulfur dioxide, but when we opened the door of the chamber, the ammonia would knock you off your feet! So I wonder what we're talking about when we say the animals are exposed to 5 ppm SO_2 if

there is all this absolutely horrible smell in the exposure chamber. One particularly notorious case was the exposure of dogs. We may have 5 ppm SO_2, but we also have a million other things. So, cleanliness in the chamber, I think, is number one. We shouldn't forget about that – and please don't tell me that you have a control chamber with the same conditions; that is not a proper excuse.

INHALATION OF RADIONUCLIDES AND CARCINOGENESIS

115 yoen

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ABSTRACT

It was not suggested until 1921 that the high incidence of lung cancer among central European pitchblende miners might be related to ionizing radiation in the mines Arguments supporting this relationship have been strengthened by recent epidemiological studies that convincingly demonstrate an increased incidence of lung cancer among uranium miners of the Colorado plateau and fluorspar miners of Newfoundland, who have been exposed to high levels of the radioactive decay products of radon. However, attempts to confirm the carcinogenic action of inhaled radon decay products in experimental animals have met with little success This has given rise to the consideration that other constituents of uranium mine environments, and perhaps cigarette smoking, may be synergistically involved — Pulmonary neoplasia has been induced in experimental animals after inhalation, intratracheal deposition, or implantation of a variety of other radionuclides, including the alpha emitters – ^{210}Po, ^{238}Pu, and ^{239}Pu – and the beta-gamma emitters – ^{103}Ru, ^{106}Ru, ^{198}Au, ^{32}Cr, ^{59}Fe, ^{35}Ba, and ^{144}Ce Studies with these radionuclides are especially relevant to the potential exposure of various segments of the human population to products of the nuclear age. Still lacking, however, is knowledge of the basic processes involved in radiation carcinogenesis, which would aid in extrapolating the results of animal experiments to man. Although inhaled radionuclides are clearly carcinogenic, much remains to be learned of their possible interrelationships with other environmental factors in contributing to the human lung cancer problem.

In 1944, Egon Lorenz, senior biophysicist for the National Cancer Institute, published a paper on Radioactivity and Lung Cancer [1] At that time, there was an increasing amount of radium being used in industries, such as in the manufacture of luminous paint, and there was increasing work in laboratories with artificially produced radioactive substances Being concerned by the reports of radon-induced lung cancer in the miners of Schneeberg and Joachimsthal, Dr. Lorenz addressed himself to two questions "What, then, is the evidence of the

carcinogenic properties of radiations with respect to lung tissue?" and "Is there sufficient evidence to warrant the statement that cancer of the lung can be induced in man by radioactive substances?" To answer these questions, Dr. Lorenz reviewed and evaluated the literature on pulmonary cancer in the Schneeberg and Joachimsthal miners and the laboratory work which had been reported at that time. Because there was no convincing evidence that radium or radon had ever induced lung cancer in man outside of the Schneeberg and Joachimsthal mines, because reports of radon-induced lung cancer in experimental animals were inconclusive, and because there was no report in the literature that lung cancer had been observed after radiation damage to the lung by either X- or γ-rays, Dr. Lorenz concluded that radon was not the sole cause of the lung cancer in the miners. In this regard, he mentioned a number of possible contributing factors in addition to radiation: pneumoconiosis produced by dust in the mines, chronic irritation caused by respiratory diseases, arsenic, and a possible hereditary susceptibility.

Twenty-five years later the situation has clarified to the extent that we can answer the two basic questions to which Dr. Lorenz addressed himself. In the past 20 years, we have developed convincing evidence for the carcinogenic potential of radioactive substances deposited in the lung of experimental animals. Also, a number of epidemiological studies of selected human occupational populations exposed to radon and radon decay products strongly suggest that these radioactive substances can cause lung cancer in man. However, there is no evidence linking lung cancer to the exposure of humans to any other inhaled radionuclide, although such exposures in the nuclear industry have been reported.[2,3] Nevertheless, the uranium miner data and the experimental animal data are convincing enough to cause many to believe that inhaled radionuclides are among the most carcinogenic substances that might be deposited in the lung. Consequently, there have been a number of attempts to implicate radionuclides as an important etiological factor in the observed high incidence of lung cancer in the population at large. Before reaching such a conclusion, it would seem prudent to examine carefully the evidence for the carcinogenicity of inhaled radionuclides. In most cases, the demonstration of the carcinogenic action of radionuclides has required that lung tissue be exposed to doses of radiation great enough to cause severe cellular damage. Relatively low doses of radiation to lung tissue generally have not proved to be carcinogenic in experimental animals. In regard to the human epidemiological studies, there are still indications that radiation may be only one of several carcinogenic agents involved.

This report will attempt to update Lorenz's 25-year-old evaluation of the carcinogenesis of radionuclides deposited in the lung. It will not be a review of the literature but will draw upon information which has appeared in a number of reviews of this general subject area.[4-11] The term *lung cancer* will be used in its broadest meaning to include all types of malignant primary pulmonary neoplasia.

HUMAN DATA

European Uranium Miners

It is appropriate to begin this discussion with a brief summary of the human mortality data which aroused the concern of Lorenz and many others. Beginning in the 15th and 16th centuries, copper, iron, silver, cobalt, arsenic, bismuth, and nickel ores were mined in the Erzgebirge (Ore) Mountains on the border between Germany and present day Czechoslovakia.[1,12,13] Pitchblende was mined for uranium and eventually radium beginning with the latter part of the last century. The two mining districts in this region, Schneeberg in Germany and Joachimsthal in Czechoslovakia, became infamous for the high incidence of a fatal pulmonary disease called "Bergkrankheit" or mountain disease by the miners.

The disease among the Schneeberg miners was diagnosed as lung cancer in 1879 by Härting and Hesse[14] and thus began a series of studies to document the incidence of the disease among the miners and other employees of the mines. However, it wasn't until the 1920's that the similarity between the diseases of the Joachimsthal and Schneeberg miners was recognized. Examples of the incidence of lung cancer in these miners are shown in Table 1. During the period 1875 to 1912 approximately 40% of the Schneeberg miner deaths were due to lung cancer. The incidence was thought to be even higher, 50% and over, during the early part of this century, which coincides with the mining of pitchblende for uranium and radium. Unfortunately, thorough epidemiological studies were not done. The miners and appropriate controls were not studied for extended periods, autopsies were infrequent, and the causes of death were often mistaken. Yet, the incidence of lung cancer and other respiratory diseases among the miners was obviously high. (Currently in the U.S., about 3% of all male deaths are due to lung cancer.)

TABLE 1

Lung Cancer in Schneeberg and Joachimsthal Miners

Location	Period of study	Number of miners	Number of miner deaths studied	Number having lung cancer	Percent lung cancer	Reference
Schneeberg	1875–1912		665*	276	41.5	15
	1922–1925	154	21	13	61.9	16
		353 (nonminers)†		2		
	1936–1939	70	6	5	83	17
Joachimsthal	1928–1938		89	43	48.3	18

*119 died of other lung diseases.

†Factory workers and residents of surrounding county.

In 1921, Uhlig mentioned the possible relationship between lung cancer and the radon content of the air in the Schneeberg mines [19] Ludewig and Lorenser[20] made the first detailed measurements in the Schneeberg mines in 1924 and reported the radon content to be 3.6×10^{-10} to 1.8×10^{-8} Ci/liter This report strengthened the idea that radioactivity was associated with lung cancer among the miners Subsequent measurements by Rajewsky[21] showed levels as high as 5.4×10^{-8} Ci/liter in an abandoned mine referred to as the death mine by the miners, but average values in operating mines were about 3×10^{-9} Ci/liter of air Holaday[22] examined these data and concluded that the levels in the operating mines ranged between 10 and 180 working levels Since the average duration of exposure associated with lung cancer in the Schneeberg and Joachimsthal miners was 17 years,[23] it can be estimated that the average exposure ranged between 2040 and 36,700 cumulative working level months (CWLM) [The working level (WL) is equivalent to 1×10^{-10} Ci ^{222}Rn/liter *] In a study of existing lung models, Parker concluded that 1 WLM would result in an exposure of about 7 rads to the nuclei of the basal cells of the bronchial epithelium [25] Therefore, the dose to the bronchial epithelium of these miners ranged between 14,000 and 257,000 rads This is probably a conservative estimate because the miners worked longer than 40-hr weeks and the concentration of radon and daughter products was often greater than 3×10^{-9} Ci/liter In addition to the radon and radon decay products, the miners inhaled large quantities of dust, as much as 6 g in 7 hr,[14] composed of silica, arsenic, cobalt, etc Evidence of the pulmonary deposition of large quantities of dust was seen in lung and bronchial lymph nodes at autopsy The mines were also very damp and cold and were in general very poor working environments Thus, while radon has been considered to be a critical factor in the development of lung cancer in these miners, it has been difficult to minimize the possible role of other factors [1,12]

Fluorspar Miners

Beginning about 1933, lung cancer deaths were reported among the fluorspar miners of St Laurence, Newfoundland [26] The fluorspar (CaF_2) mining industry, like the uranium mines of Europe, employed miners from a relatively stable population Like the European uranium mines, the fluorspar mines are very damp and cold Radon and radon decay products were discovered in the mines and are thought to originate in the water seeping into the mines The concentration of radon in the dead end areas of the mines ranged from $2.7 \times$

*A working level (WL) is defined as any combination of short lived radon daughters in 1 liter of air that will result in the ultimate emission of 1.3×10^5 Mev of potential alpha energy Inhalation of air with a concentration of 1 WL of radon daughters for 170 working hours results in an exposure of 1 working level month (WLM) [4] The short lived ^{222}Rn daughter products contribute the principal radiation dose to the lungs [24]

TABLE 2

Lung Cancer in Fluorspar Miners (1933 to 1961)[26]

	Number of employees	Number of deaths	Deaths due to lung cancer	Percent due to lung cancer
All employees	2000	119	26	21.8
Underground employees	630	69	25	36.2

10^{-10} to 2.5×10^{-8} Ci/liter and in the ventilated areas, from $< 5 \times 10^{-12}$ to 1.5×10^{-9} Ci/liter.

The incidence of lung cancer is summarized in Table 2. Of about 2000 employees, 630 worked underground. In a total of 119 deaths occurring between 1933 and 1961, 26 were due to lung cancer. All but one of these occurred in the underground miners. The incidence of lung cancer among the underground miners was about 36%. The miners worked from 5.5 to 21.3 years underground, the average being about 12.5 years, and were probably exposed to an average radon daughter concentration of 2.5 to 10 WL.[26] Thus, the average total exposure of the miners could range from 165 CWLM for those working 5.5 years to 2556 CWLM for the 21.3-year employee. Evans estimates that the average total exposures of the miners working 20 years ranged from 800 to 2400 CWLM.[27] The average radiation dose to the bronchial epithelium of these workers could have ranged from 1000 to 18,000 rads, but the maximum exposures could have been much higher for miners working for extended periods at the highest measured radon concentrations of 10^{-8} Ci radon per liter (100 WL).

The miners were exposed to quartz and fluorspar (CaF_2) dust in the mines, but measurements made in 1956 and 1957 did not show high concentrations. Fluoride levels were below the threshold limit values. Apparently a large fraction of the miners were smokers – 18 of 20 for whom the information was available.[26] Thus, while the preponderance of the evidence points to radon and radon decay products as the cause of lung cancer in the fluorspar miners, there are other possible etiological factors to be considered.

U.S. Uranium Miners

The uranium mining industry in the U.S. was a minimal effort until after about 1946 when extensive ore deposits were discovered in the Colorado Plateau. As the industry expanded, there was concern for the potential health hazards to the miners working in the more than 350 mines scattered over a 140,000 square mile area. Most of the mines employed less than 10 men. Dire consequences were predicted – for example, in 1954, Hueper[28] stated that "since the mines had been in operation less than 10 years, it was probably too early for appreciable numbers of lung cancers to develop, but the exposure

TABLE 3

Lung Cancer in U.S. Uranium Miners (1950 Through 1967)[31]

	Number of employees in study	Number of deaths	Deaths due to lung cancer	Percent due to lung cancer
White miners	3414	398*	62	16
Nonwhite miners	761	59†	2	3.4

*120 violent deaths.
†30 violent deaths.

conditions are such that the occurrence of radiation cancers of the lung may be anticipated for the coming years." In 1963, Wagoner *et al.*[29,30] reported a higher than expected incidence of lung cancer among miners with long-term underground employment. An extensive epidemiological study has been updated recently by Lundin *et al.*[31] (Table 3). Through September 1967, there were 62 cases of lung cancer in 3414 white miners and two cases in 761 nonwhite miners. Lung cancer accounted for 16% of the deaths of the white miners and 3.4% of those of nonwhite miners. These were distributed in time as shown in Fig. 1. The first case appeared in 1955. Ten years later, in 1965, there were 17 cases. In 1968, only one miner death was due to lung cancer. The peak year for lung cancer was about 18 years after uranium mining activities were expanded in the Colorado Plateau area. Extensive studies of this mining population have provided relatively complete occupational, medical, and personal histories. From estimates of atmospheric concentrations of radon daughters in the uranium mines and the work history of the miner, the cumulative radiation exposure of each miner was calculated and expressed in terms of the cumulative working level month (CWLM). These data have been compiled by Lundin *et al.*[31] The radiation exposure in CWLM is plotted against the age of the miner at death in Fig. 2. The work history of each miner is also shown graphically. Years of hard rock mining, years of exposure in uranium mines, and the years which elapsed from the conclusion of his exposure in the uranium mine until death are indicated on a "life line" for each miner.

Estimated exposures of these miners ranged from 5 to almost 7000 CWLM, which may be equivalent to doses of 35 to 49,000 rads to the bronchial epithelium. Nearly all of the lung cancer deaths occurred after the age of 45. In many cases death occurred after several years absence from the uranium mines. A large number of the miners had many years of hard rock mining prior to their uranium mine experience. This was especially true for those in the lower WL exposure range. Only two of the miners were nonsmokers. All of the rest smoked from 1 to 2½ packs of cigarettes per day, except for about four, who smoked one-half pack per day.

Dr. Lundin[31] has also reported these cancer deaths in terms of expected cancer deaths in the male populations of the states in which the miners worked.

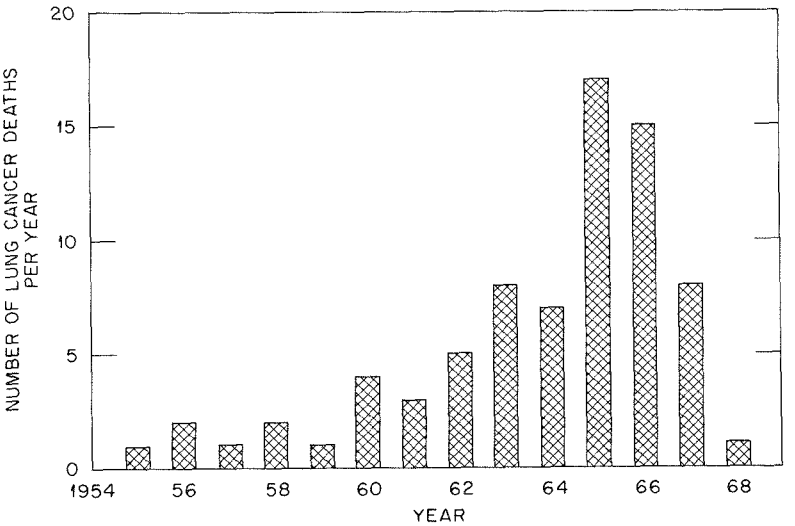

Fig. 1 – Yearly occurrences of lung cancer among U.S. uranium miner deaths. (Prepared from data published by Lundin *et al.*[31])

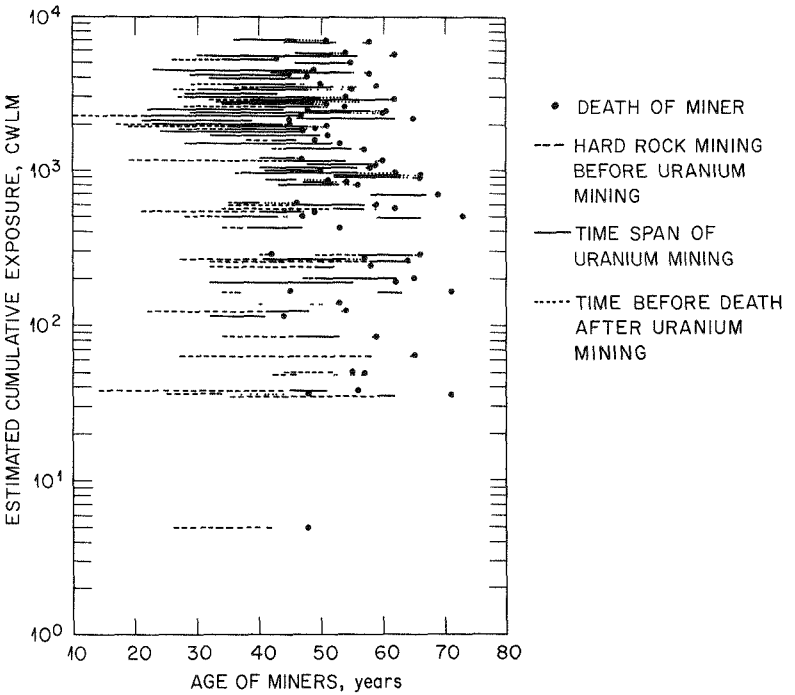

Fig. 2 – Mortality and mining experience of U.S. uranium miners. (Prepared from data published by Lundin *et al.*[31])

TABLE 4

Expected and Observed Respiratory Cancer Deaths in White Uranium Miners (1950 to 1967)[31]

Estimated cumulative exposure (CWLM)	Person-years at risk	Lung cancer deaths	
		Expected	Observed
<120	10,325	2.51	8*
120–359	9,554	2.39	10*
360–839	7,368	2.27	7†
840–1799	5,107	1 70	11*
1800–3719	2,406	0.95	17*
>3720	679	0.24	9*
Total	35,439	10.06	62*

*Significant at 1% level.
†Significant at 5% level.

These data are summarized in Table 4, which shows the expected and observed respiratory cancer deaths in relation to the estimated cumulative exposure to radon daughter products These data show a statistically significant excess of lung cancer deaths in each of the three categories of estimated cumulative exposures under 840 CWLM In the exposure categories above 840 CWLM, the lung cancer risk increased with exposure These data were interpreted by a National Academy of Sciences–National Research Council (NRC-NAS) Advisory Committee[8] as providing strong evidence for the hypothesis that radiation is a causal factor in lung cancer at these exposure levels. The data from the exposure categories below 840 CWLM are more difficult to interpret because of the small number of cases, possible inaccuracies in the exposure data, and the unknown factor of prior hard rock mining which a large number of the miners in these groups experienced It is also known that miners are exposed to diesel exhaust fumes, products of the detonation of explosives and dust composed of silica and a variety of metal ores The NRC-NAS Advisory Committee as well as other experts believe that the data suggest cigarette-smoking miners are particularly susceptible to lung cancer. Saccomanno, after extensively studying the pulmonary pathology of the Colorado plateau uranium miners, is convinced of a synergism between cigarette smoking and radon daughters in causing lung cancer Using the results of epidemiology studies, he compares the relative incidence of lung cancer among uranium miners, smokers, and nonsmokers (Fig 3)[32] On the basis of 100,000 population, 700 lung cancer deaths per year would occur among cigarette-smoking uranium miners, compared with four among nonsmoking miners Comparative data are also shown for smoking and nonsmoking nonminers.

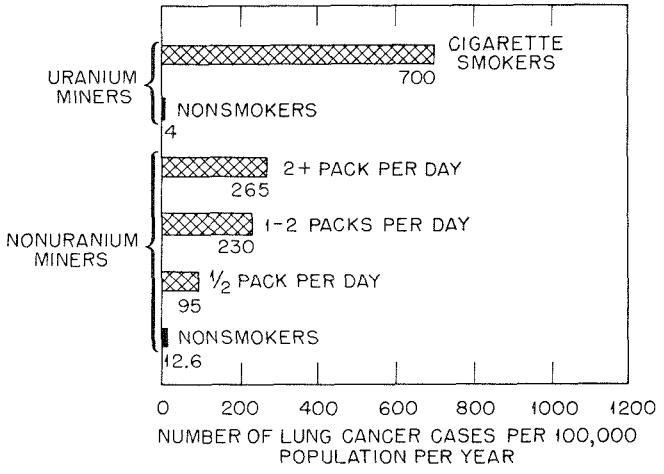

Fig. 3 – Relative incidence of lung cancer in uranium miners, smokers, and nonsmokers. (Redrawn from Saccomanno[32])

The epidemiology data are strongly suggestive that radiation exposure contributed to the excess lung cancer among the Colorado Plateau uranium miners. The cumulative exposures of these miners were probably less than experienced by the Schneeberg and Joachimsthal miners, but possibly greater than the average exposures of the fluorspar miners. The data do not permit the exclusion of other possible contributing factors encountered by the miners in their working environment, or the possible role of cigarette smoking.

ANIMAL STUDIES

In 1944, Lorenz reviewed the studies which attempted to determine whether inhalation of radon would cause lung cancer in experimental animals. Two kinds of studies had been done. Animals – mice and rats – were either exposed in the mines of Schneeberg and Joachimsthal, or were exposed to radon in the laboratory.[1] In many of these studies early mortality resulted from respiratory tract infections or, in some cases, where radon levels were high, from radiation injury, but not from radiation injury to lung tissue. Although some of the authors reported an increased tumor incidence, Lorenz concluded there was no evidence that radon could induce lung tumors in animals.

Additional studies have been completed since 1944 in which either radon alone or in combination with its decay products and other constituents of uranium mine air were administered by inhalation to experimental animals.[33-37] Even though a few tumors were reported, the results do not support conclusions different from those drawn earlier by Lorenz. Other studies now in progress in France, Yugoslavia, and the U.S., emphasizing possible synergistic action of various constituents of uranium mine environments and cigarette smoking, may

TABLE 5

Summary of Radionuclide-Induced Lung Cancer in Experimental Animals

Inhaled* or injected particles						Implanted sources					
			Lung cancer						Lung cancer		
Radionuclide	Species	Number	Number	Percent	Reference	Radionuclide	Species	Number	Number	Percent	Reference
^{210}Po α	Rat*	380	32	8	40	^{106}Ru β-γ	Rat	191	75	39	51, 52
	Rat	15	2	13	41		Rat	177	66	37	53
^{239}Pu α	Mice	17	2	12	42		Rat	16	13	81	54
	Mice			12	39	^{32}P β	Rat	48	15	31	52
	Dog*	26	18	70	43	^{90}Sr β	Rat			90	55
	Rat*			50–100	44		Rat	23	4	17	56
^{144}Ce β-γ	Rat				38	^{60}Co β-γ	Mice	190	20	11	57
	Rabbit	20	5	25	45		Mice	286	57	20	58
	Rat	435	112	26	46		Rat	20	15	75	58
^{106}Ru β-γ	Mice	21	2	5	42		Hamster	25	2	8	58
^{32}P β	Rat	76	11	14	47		Guinea Pig	20	5	25	58
	Rat			4	48		Rabbit	12	5	42	58
^{198}Au β-γ	Rat	30	3	10	47						
^{59}Fe β-γ	Rat	52	8	15	47						
	Rat				49						
^{35}S β	Rat	16	2	13	50						
^{103}Ru β-γ	Rat			6	48						

show why the lung cancer observed in uranium miners has not been producible in experimental animals.

Although attempts to induce lung cancer with inhaled radon and radon decay products in experimental animals have failed, success has been achieved with other radioactive materials. In 1949 Lisco and Finkel reported malignant tumors in rats after inhalation of 3.2 to 200 μCi $^{144}CeO_2$.[38] Details were not published, but the abstract noted the nonuniform distribution of ^{144}Ce in the lungs. Wager *et al.*, in 1955, reported lung cancer induction in mice with 0.06 μCi $^{239}PuO_2$ given by intratracheal administration.[39] Subsequently, a number of laboratories have experimentally induced lung cancer in several animal species with several radionuclides, but a tabulation of all the reported data is not impressive in terms of numbers of studies or numbers of tumors observed (Table 5). This table lists most of the published experiments. Qualitative aspects of these studies will be described in this paper. The quantitative relationships can be found elsewhere in these proceedings (see C. L. Sanders *et al.*, p. 285).

Most of those working on the problem would agree that the induction of lung cancer in experimental animals is not an easily accomplished or predictable laboratory procedure. Table 5 is misleading in that none of the unsuccessful experiments are listed. To demonstrate pulmonary carcinogenesis, the amount of material deposited and the resultant radiation dose rate must not cause death before a tumor can become manifest but must be adequate to produce a tumor within the normal lifetime of the animal. This requirement is not easily achieved, especially by exposing animals to radioactive aerosols.

Techniques have been developed to circumvent this problem. For example, the group at New York University modified their technique for implanting pellets containing chemical carcinogens in the lung. ^{106}Ru-plated platinum pellets implanted in the distal segment of the bronchi of rats[52] delivered 10^2 to 10^6 rads beta radiation to the basal cells of the bronchial epithelium. Because the high dose delivered by this technique was localized, sufficient respiratory tissue remained functional to sustain the animal for the major part of its normal life-span or until death occurred as a result of neoplasia. This study also illustrates a threshold dose response for the induction of lung cancer (Fig. 4).[52] Doses above about 3×10^5 rads were required for tumor induction. Of about 400 rats treated by this technique, nearly 40% developed squamous cell carcinoma. A similar technique was used by another laboratory to develop a tumor incidence of about 80%.[54]

Another technique for irradiating lung tissue with radioactive implants was developed by Warren and Gates.[57,58] Bronchogenic carcinomas and epidermoid carcinomas occurred in mice, rats, hamsters, guinea pigs, and rabbits which were given lung implants of ^{60}Co wire (Fig. 5). Mean total gamma radiation doses to tissues adjacent to the wire ranged from 2.6×10^5 to 9.1×10^5 R. The rat was the most susceptible species for induction of lung cancer. The gamma radiation from the ^{60}Co wire also caused bone sarcomas and cancer of the esophagus in a large number of the animals. This study is especially important because it

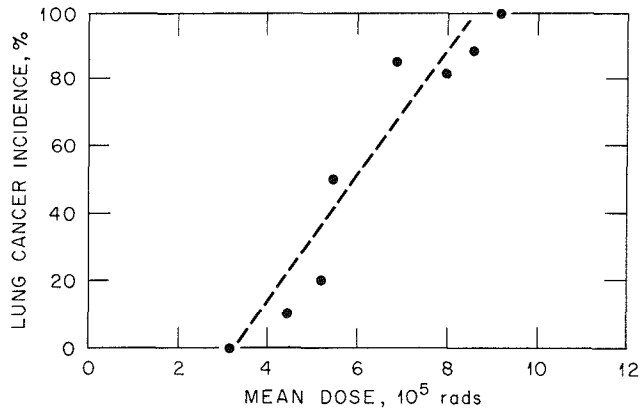

Fig. 4 – Lung cancer in rats exposed to ^{106}Ru-^{106}Rh pellet implants. (Prepared from data published by Laskin *et al.*[52])

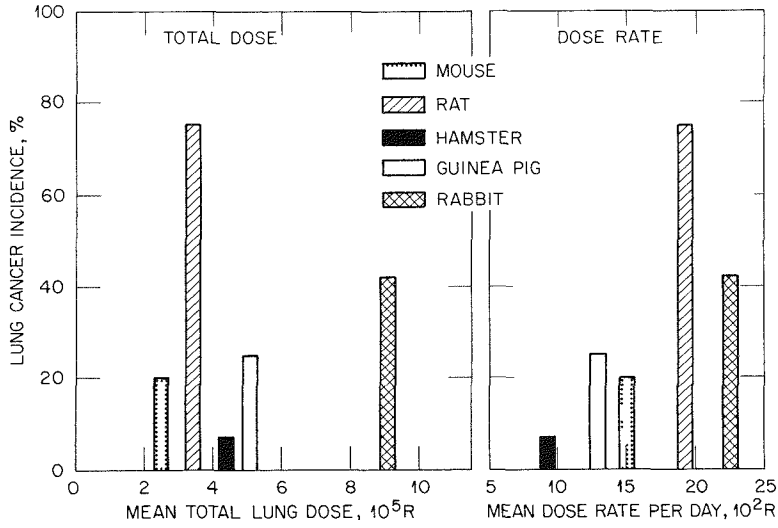

Fig. 5 – Lung cancer in animals exposed to ^{60}Co wire implants. (Prepared from data published by Warren and Gates[53])

compared the responses of several animal species, thus providing an approach to extrapolating experimental animal data to man.

Cember[5,46] has very successfully utilized intratracheal insufflation of radionuclides to produce lung cancer in rats. It can be argued that this technique is an unnatural route for entry of radioactive particles to the lung and results in greater nonuniformity of deposition than occurs after inhalation. Because it is less traumatic than the implantation of pellets and wire, however, the results from such experiments are probably more applicable to problems of inhaled materials. Cember treated over 400 rats with 0.5 to 50 μCi $^{144}CeF_4$ or

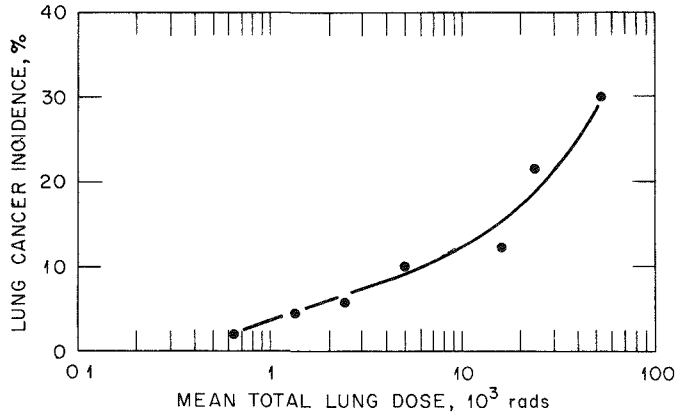

Fig. 6 – Lung cancer in rats given ^{144}Ce by intratracheal injection. (Redrawn from Cember[46])

$^{144}CeCl_4$ and found neoplasia in the lungs of 112 The integrated dose to the lungs of the rats showing tumors ranged from 600 to about 50,000 rads and was logarithmically related to cancer incidence (Fig. 6) A threshold dose response was not demonstrated in these experiments Other authors have reported pulmonary neoplasia after intratracheal administration of several radionuclides (Table 5) Thus, intratracheal administration has been a useful technique to test the carcinogenic properties of radionuclides.

Relatively few studies have been done in which radionuclides were deposited in the respiratory tract by inhalation Other than the early work by Lisco on ^{144}Ce (ref. 38), only three studies showed evidence of lung cancer, and all of these involved alpha-emitting radionuclides. Current studies of inhaled beta-gamma emitters in dogs at two laboratories have not yet shown pulmonary neoplasia. The most effective radionuclide for inducing lung cancer in experimental animals was ^{210}Po. Primary lung cancer was first observed in rats at 2 months after exposure to a NaCl aerosol containing ^{210}Po (Fig 7)[40] Three percent of the animals in the low dose groups had primary lung cancer, and it occurred in 10 and 13% of the high dose groups This study is particularly important because lung tumors occurred in animals which received a total average lung dose of 71 rads However, at least two factors need to be considered in assessing the significance of these findings. The radiation dose calculated for the rats is based on the assumption that the energy from the alpha radiation was uniformly absorbed throughout the lung tissue and not localized. Second, acute and chronic murine type pulmonary infection was endemic in the rats and was the most common cause of death. The possibility of these lesions being contributory to the carcinogenic action of the alpha radiation needs to be determined.

In another study involving $^{239}PuO_2$ inhaled by dogs, pulmonary neoplasia was found in 18 of 26 dogs which had died up to about 9 years after

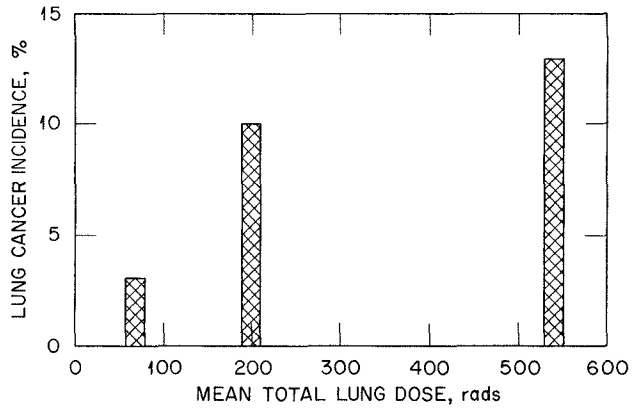

Fig. 7 – Lung cancer in rats after inhalation of ^{210}Po. (Prepared from data published by Yuile *et al.*[40])

exposure.[43] The data from this experiment and an early higher dose study provide an example of the concept of a practical threshold for radionuclide-induced life shortening and carcinogenesis as discussed by Evans in regard to human radionuclide exposure.[59] Although one might be inclined to believe that there is a certain radiation dose below which cancer is not induced, the available data are not conclusive. Most radiation biologists hold the more conservative view that the relationship between cancer incidence and radiation dose is linear. However, it is conceivable that at very low radiation doses, the cancer induction or appearance time might exceed the normal life-span of the individual. This low dose would be a practical threshold dose. Figure 8 illustrates this possibility. The dogs were given a single exposure to an aerosol of ^{239}Pu. The amount of ^{239}Pu deposited in the alveolar region of the lung is plotted against the time of death for each dog. Animals with relatively large deposits of ^{239}Pu in their lungs died early owing to radiation injury of the functional tissue of the lung. Death, due to the same cause, occurred late enough in others for primary lung cancers to become manifest. Several dogs which inhaled small amounts of plutonium died at longer times after exposure as a result of lung neoplasia. Shown on the figure is a vertical line identifying 15 years as being about the expected life-span of a beagle dog. The curve fitted to the data by least squares analyses can be extrapolated to 15 years to obtain an estimate of the minimum amount of ^{239}Pu which might be expected to cause a premature death, probably due to lung cancer in this case. This value appears to be less than 5 nCi/g of lung and is in a sense, a practical threshold for the induction of lung cancer in beagle dogs by inhaled $^{239}PuO_2$.

Animal experimentation has helped to identify another aspect of the problem of inhaled radionuclides which needs to be considered in a discussion of carcinogenesis. The translocation of radionuclides from the lung may result in higher concentrations of radionuclides in other tissues than in the lung. For

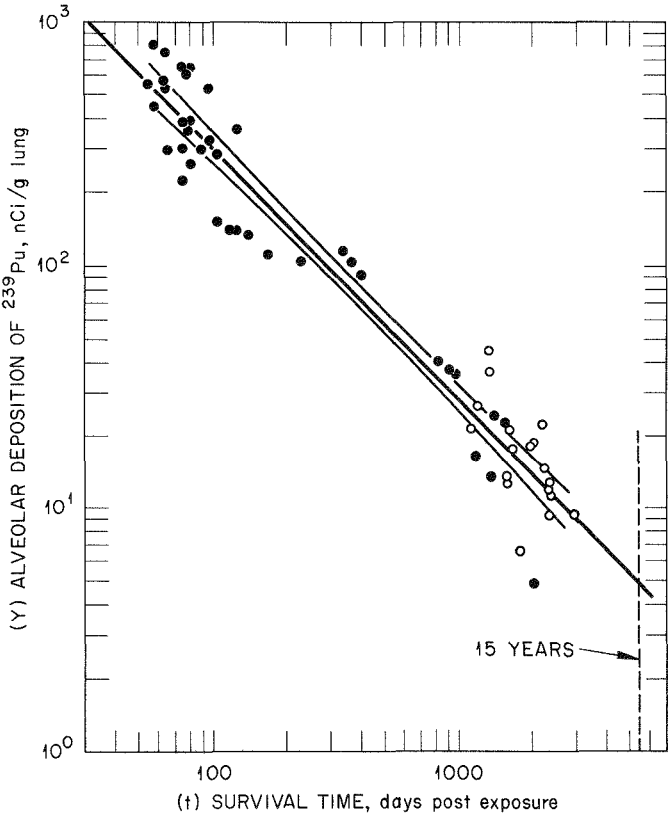

Fig. 8 – Relationship between quantity of $^{239}PuO_2$ deposited and survival time of dogs.[43]

example, if the inhaled radionuclide compound is soluble, it may accumulate in bone, liver, kidney, etc. If the radionuclide is relatively insoluble, major accumulation outside the lung will be in the tracheobronchial lymph nodes. An example of this is shown in Fig. 9, which summarizes the retention and translocation of inhaled $^{239}PuO_2$ in beagle dogs over a period of about 8 years after exposure. During this period, although there was continuous excretion of ^{239}Pu from the body, the major fraction of the plutonium which cleared the lung accumulated in the tracheobronchial lymph nodes. The average concentrations of plutonium in these lymph nodes were 50 to 100 times that of the lung. If both tissues are equally susceptible to radiation-induced cancer, it would be expected that neoplastic changes in the lymph nodes would occur before they occurred in the lung, but this has not been our experience to date. Although metastasis from the lung tumors has occurred, none of the studies on radionuclide-induced lung cancer have reported primary neoplasia in the associated lymph nodes, with the exception of a lymphangiosarcoma in the lung

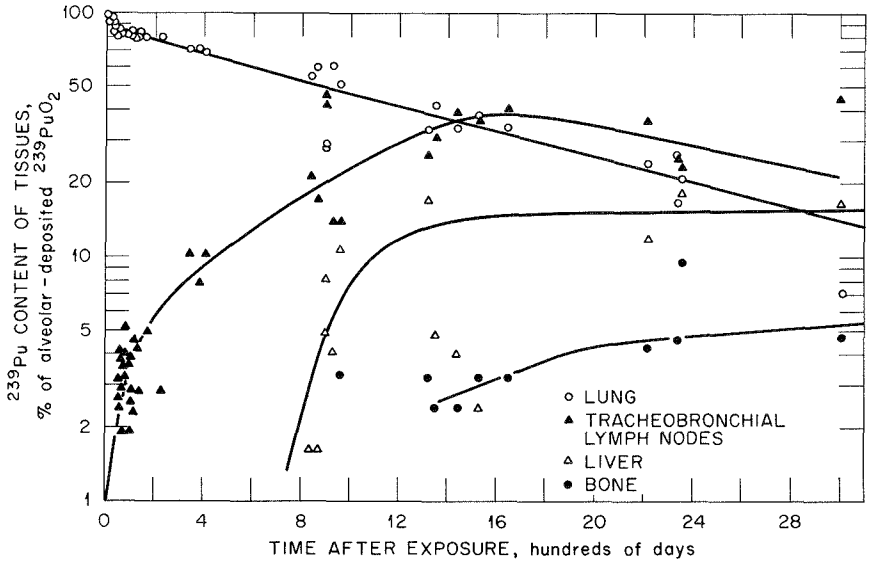

Fig. 9 – Retention and translocation of alveolar-deposited $^{239}PuO_2$ in dogs.[43] CMD = ~0.4 μm; MMD = ~3 μm.

of one dog and in the lymph node of another dog after inhalation of $^{239}PuO_2$.[60] Both dogs also had primary pulmonary neoplasia. Further research is urgently needed to evaluate the relative susceptibility of lymph node and lung tissue to the carcinogenic action of radionuclides.

EXTRAPOLATION OF ANIMAL DATA TO MAN

The experimental animal studies have clearly demonstrated the carcinogenicity of radionuclides deposited in the lung. Though we are tempted to extrapolate these results to man, at least qualitatively, we are well advised to exercise caution, because radon and radon decay products have not induced lung cancer in experimental animals, yet are strongly suspect as being the cause of lung cancer in miners. Extrapolation of experimental animal data to man on a quantitative basis can be even more misleading because a common denominator for comparing radiation doses to lung tissue has not been identified. Thus, it is difficult to relate the doses estimated for the bronchial tissues of the uranium miners to the doses calculated for the experimental animals. It would seem urgent that the next generation of experiments be directed toward this problem. Factors which need evaluation are the relative susceptibilities of human and experimental animal tissues to radiation-induced cancer, the relative latent periods for the induction of cancer in man and other species, possible species differences in the rates of clearance and translocation of inhaled radionuclides, and a number of other factors which pertain to the still unknown mechanisms of

tumor induction. A serious obstacle to evaluating results of animal experiments with inhaled radionuclides and extrapolating them to man is the difficulty in identifying the effective biological target tissue in the lung and measuring the radiation dose to that tissue.

Possibly a major factor in extrapolating the results of animal experiments to man is the fact that experimental animals do not come into contact with many of the air pollutants to which man is exposed, not only in his work environment but at home as well. Of course, smoking is already suspect as being an important contributing factor to the induction of lung cancer. In this regard, it is of interest to note that the experimental animal work of Yuile *et al.*[40] is being cited to support the argument that extremely small amounts of ^{210}Po in tobacco smoke may be an etiological factor in the genesis of lung cancer in cigarette smokers.[61,62] A number of studies which may contribute to solving the problem of extrapolating animal data to man will be discussed at this meeting. It is hoped that other studies will be initiated in the near future.

CONCLUSION

The objective of this paper was to update Lorenz's 25-year-old evaluation of the carcinogenesis of radionuclides deposited in the lung. Lorenz concluded that inhalation of radon was not the sole cause of lung cancer in uranium miners, primarily on the basis that X-ray doses of more than 1000 R had not produced lung tumors in experimental animals. Today, although there is still no conclusive experimental evidence that inhaled radon and radon decay products will cause lung cancer, the case is stronger for indicting radiation as a major cause of lung cancer in uranium miners. The evidence includes experimental animal data with other radionuclides, which clearly demonstrate that ionizing radiation can induce lung cancer, and extensive epidemiology studies on fluorspar and uranium miners. However, the case is also stronger for factors other than radiation being contributory in either initiating or promoting carcinogenesis. This is particularly true of cigarette smoking.

As a result of the rapid expansion of the atomic energy industry over the past 25 years, uranium miners no longer constitute the major population potentially exposed to airborne radionuclides, and radon decay products do not predominate as the radionuclides of most concern. In contrast to radon and radon decay products, the carcinogenic properties of a number of the important radionuclides associated with the nuclear industry have been conclusively demonstrated in experimental animals. The need now is to understand the mechanism of the carcinogenic action of radionuclides, to define the sensitive tissues and the dose response relationship particularly at the low dose levels likely to be encountered by the human population, and to determine possible cocarcinogenic relationships between radiation and other environmental factors. Finally, progress is needed in developing more valid bases for extrapolating the results of animal experiments to man.

ACKNOWLEDGMENTS

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REFERENCES

1 E Lorenz, Radioactivity and lung cancer A critical review of lung cancer in the miners of Schneeberg and Joachimsthal *J Natl Cancer Inst* 5 (1944) 1 15
2 D Newton, A case of accidental inhalation of protactinium-231 and actinium 227 *Health Phys*, 15 (1968) 11–17
3 J R Mann and R A Kirchner, Evaluation of lung burden following acute inhalation exposure to highly insoluble PuO_2 *Health Phys* 13 (1967) 877–882
4 Federal Radiation Council, Guidance for the Control of Radiation Hazards in Uranium Mining, Staff Report No 8, Revised Superintendent of Documents, U S Government Printing Office, Washington, D C , September 1967
5 H Cember, Progress in radiogenic lung cancer In, *Lung Tumors in Animals* pp 321–330, L Severi (ed) University of Perugia, Italy, 1966
6 C L Sanders, R C Thompson, and W J Bair, Carcinogenesis in the Lung from Inhalation of Radioactive Particles with Special Reference to Hazards of Nuclear Rocket Engines, USNRDL Report, TRC-69-8 United States Naval Radiological Defense Laboratory, San Francisco, California, May 1969
7 N Nelson, Radiation cancer of the lung tissue sensitivity and factors influencing dosage from airborne radioactive substances In, *Inhaled Particles and Vapours* pp 221–228, C N Davies (ed) Pergamon Press, Oxford, 1961
8 Federal Radiation Council, Radiation Exposure of Uranium Miners, Report of an Advisory Committee from the Division of Medical Sciences, National Academy of Sciences – National Research Council – National Academy of Engineering, Washington, D C , August 1968
9 Report of the Subcommittee on Inhalation Hazards, Effects of Inhaled Radioactive Particles, Publication No 848. National Academy of Sciences, National Research Council, Washington, D C , 1961
10 J Furth and J L Tullis, Carcinogenesis by radioactive substances *Cancer Res* 16 (1956) 5–21
11 R Seltser, Lung cancer and uranium mining – a critique *Arch Environ Health,* 10 (1965) 923–936
12 C V Weller, *Causal Factors in Cancer of the Lung* Charles C Thomas, Springfield, Illinois, 1956
13 A Pirchan and H Sikl, Cancer of the lung in the miners of Jachymov (Joachimsthal) Report of cases observed *Am J Cancer* 16 (1932) 681 722 Cited by Lorenz [1]
14 F H Harting and W Hesse, Der Lungenkrebs, die Bergkrankheit in den Schneeberger Gruben *Vierteljahrsschr gerichtl Med offenth Gesundheitswesen,* (N F), 30 (1879) 296–309, 31 102–129, 313–337 Cited by Lorenz [1]
15 A Arnstein, Sozialhygienische Untersuchungen uber die Bergleute in den Schneeberger Kobalt-Gruben, insbesodere uber das Vorkommen des sogenannten "Schneeberger Lungenkrebses" *Osterreich Sanitatswesen v 38, Wien Arbeit a d Geb d Soz Med Beihefte* 5 (1913) 64–83 Cited by Lorenz [1]
16 O Rostoski, E Saupe, and G Schmorl, Die Bergkrankheit der Erzbergleute in Schneeberg in Sachsen ("Schneeberger Lungenkrebs") *Z Krebsforsch* 23 (1926) 360–384 Cited by Lorenz [1]

17 W Hueck, Kurzer Bericht uber Ergebnisse Anatomischer Untersuchungen in Schneeberg *Z Krebsforsch,* 49 (1939) 312–315 Cited by Lorenz [1]
18 S Peller, Lung cancer among mine workers in Joachimsthal *Human Biol* 11 (1939) 130–143 Cited by Lorenz [1]
19 M Uhlig, Uber den Schneeberger Lungenkrebs *Virchows Arch Pathol Anat*, 230 (1921) 76–98 Cited by Weller [1 2]
20 P Ludewig and E Lorenser, Untersuchung der Grubenluft in den Schneeberger Gruben auf den Gehalt an Radiumemanation *Z Phys*, 22 (1924) 178 185 Also in *Strahlentherapie* 17 (1924) 428 435
21 B Rajewsky, Bericht uber die Schneeberger Untersachungen. *Z Krebsforsch,* 49 (1939) 315–340 Cited by Lorenz [1]
22 D A Holaday In, *Radiation Exposure of Uranium Miners, Hearings of the Joint Committee on Atomic Energy Congress of the United States* Part I, pp 654–655 Superintendent of Documents, U S Government Printing Office, Washington, D C , 1967
23 H Sikl, The present status of knowledge about Jackymov disease (Cancer of the lungs in the miners of the radium mines) *Acta Unio Intern Contra Cancrum,* 6 (1950) 1366–1375 Cited by C G Stewart and S D Simpson, The hazards of inhaling radon-222 and its short lived daughters Consideration of proposed maximum permissible concentrations in AIR In, *Radiological Health and Safety in Mining and Milling of Nuclear Materials* Vol 1, pp 333 357 International Atomic Energy Agency Vienna, 1964
24 J Shapiro and W F Bale, A Partial Evaluation of the Hazard from Radon and Its Degradation Products, USAEC Report, UR 242 University of Rochester, N Y , 1953
25 H M Parker, The dilemma of lung dosimetry *Health Phys*, 16 (1969) 553 561
26 A J de Villiers and J P Windish, Lung cancer in a fluorspar mining community I Radiation, dust, and mortality experience *Brit J Ind Med* 21 (1964) 94–109
27 R D Evans, Radiation protection of uranium miners In, *Radiation Standards for Uranium Mining, Hearings before the Subcommittee on Research, Development, and Radiation of the Joint Committee on Atomic Energy, Congress of the United States March 17 and 18, 1969,* Part II, pp 317–343 Superintendent of Documents, U S Government Printing Office, Washington, D C , 1969
28 W C Hueper, Recent developments in environmental cancer *Arch Pathol*, 58 (1954) 360–399, 475–523, 645–682
29 J K Wagoner, V E Archer, F E Lundin, D A Holaday, and J W Lloyd, Radiation as the cause of lung cancer among uranium miners *New Engl J Med* 273 (1965) 181–188
30 J K Wagoner, V E Archer, B E Carroll, D A Holaday, and P A Lawrence, Mortality patterns among United States uranium miners and millers, 1950 through 1962 Preliminary report In, *Radiological Health and Safety in Mining and Milling of Nuclear Materials,* Vol I, pp 37–48 International Atomic Energy Agency, Vienna, 1964
31 F E Lundin, Jr , J W Lloyd, E M Smith, V E Archer, and D. A Holaday, Mortality of uranium miners in relation to radiation exposure, hard-rock mining and cigarette smoking – 1950 through September 1967 *Health Phys*, 16 (1969) 571–578
32 G Saccomonno, Uranium miners' health In, *Radiation Standards for Uranium Mining, Hearings before the Subcommittee on Research, Development and Radiation of the Joint Committee on Atomic Energy Congress of the United States March 17 and 18, 1969,* Part I, pp 301–315 Superintendent of Documents, U S Government Printing Office, Washington, D C
33 F Unnewehr, Durch Radoninhalation Hervorgerufene Tumorbildung und Gewebsveranderung *Strahlentherapie,* 108 (1959) 421–427
34 D A Morken and J K Scott The Effects on Mice of Continual Exposure to Radon

and Its Decay Products on Dust, USAEC Report No. UR-669. University of Rochester, N.Y., 1966.

35. V. S. Kushneva, On the problem of the long-term effects of combined injury to animals of silicon dioxide and radon. In, AEC-TR-4473, pp. 22–30, 1959.
36. V. S. Kushneva, Peculiarities of the course of experimental silicosis with inhalation of radon. In, *Radiological Health and Safety in Mining and Milling of Nuclear Materials,* Vol. I, pp. 317–331. International Atomic Energy Agency, Vienna, 1964.
37. J. K. Scott, The Histopathology of Mice Exposed to Radon, USAEC Report UR-411. University of Rochester, N.Y., 1955
38. H Lisco and M. P. Finkel, Observations on lung pathology following inhalation of radioactive cerium. *Federation Proc.,* 8 (1949) 360–361.
39. R. W. Wager *et al,* Toxicity of radioactive particles In, Biology Research Annual Report for 1955, HW-41500, p. 61. Hanford Atomic Products Operation, Richland, Wash., 1956.
40. C. L. Yuile, H. L. Berke, and T. Hull, Lung cancer following polonium-210 inhalation in rats. *Radiation Res.,* 31 (1967) 760–774.
41 J. K. Scott and R. G. Thomas, Induction of pulmonary tumors following intratracheal injection of polonium-210. *University of Rochester Atomic Energy Project Quarterly Review,* July–September (1957) 30.
42. L. A. Temple, S. Marks, and W. J. Bair, Tumours in mice after pulmonary deposition of radioactive particles. *Intern. J Radiation Biol.,* 2 (1960) 143–156.
43. J F. Park, W. J. Bair, E. B. Howard, and W. J. Clarke, Chronic Effects of Inhaled Plutonium Dioxide in Dogs, USAEC Report BNWL-1050. Pacific Northwest Laboratory, Richland, Wash., in press.
44. H. Lisco, Autoradiographic and histopathologic studies in radiation carcinogenesis of the lung. *Lab Invest,* 8 (1959) 162–170.
45. N. N. Kurshakova and A. E. Ivanov, A model of experimental lung cancer caused by intratracheal introduction of radioactive cerium. *Bull. Exptl. Biol. Med.,* 54 (1962) 787–789
46. H. Cember, Empirical establishment of cancer-associated dose to the lung from ^{144}Ce, *Health Phys,* 10 (1964) 1177.
47. T. A. Kochetkova, G. A. Avrunina, and N. D. Sagaidak-Chernyak, Experimental lung cancer induced with radioactive compounds P^{32}, Au^{198} and Fe^{59}. *Acta Unio. Intern. Contra Cancrum,* 19 (1963) 684–686.
48. L A. Grizute, On the experimental cancer of the lung. In, *Proceedings of the 4th Session of the Oncological Institute, Lit SSSR, Vilnius,* p. 53, 1961. (In Russian and Lithuanian)
49. N. D. Sagaidak and M. po Toksikol, Morphological changes in rats after intratracheal introduction of various iron-59 compounds. *Radioaktivn. Veschestv Inst Gigieny Truda i Profza-bolevanu Akad. Med. Nauk SSSR,* 3 (1962) 131 146. (NSA 18, 6720, 1964)
50. H. Cember and J. A. Watson, Bronchogenic carcinoma from radioactive barium sulfate. *A.M.A. Arch. Ind Health,* 17 (1958) 230–235
51. M. Kuschner, S. Laskin, M. Daniels, and N. Nelson, The pathogenesis of radiation induced bronchogenic carcinoma in rats. In, *Lung Tumors in Animals,* pp. 369–379, L. Severi (ed.). University of Perugia, Italy, 1966.
52. S. Laskin, M. Kuschner, B. Altshuler, and N. Nelson, Tissue reactions and dose relationships in rats following intrapulmonary beta radiation. *Health Phys.,* 10 (1964) 1229–1233.
53. S. Laskin, M. Kuschner, N. Nelson, B. Altshuler, J. H. Harley, and M. Daniels, Carcinoma of the lung in rats exposed to the β-radiation of intrabronchial ruthenium-106 pellets. I Dose-response relationships, *J. Natl Cancer Inst,* 31 (1963) 219–231.

54 M B Divertie, J L Titus, and R G Shorter, A simple method for the production of experimental squamous cell carcinoma of the lung in rats *Am Rev Respirat Diseases,* 96 (1967) 820–822
55 H W Altmann, W Hunstein, and E Stutz, Strahleninduzierte (Sr^{90}) Lungencarcinome bei Ratten *Naturwissenschaften,* 46 (1959) 85 86
56 H Cember and J A Watson, Carcinogenic effects of strontium-90 beads implanted in the lungs of rats, *Am Ind Hyg Assoc J* 19 (1958) 36–42
57 O Gates and S Warren, The production of bronchial carcinoma in mice *Am. J Pathol* 36 (1960) 653 662
58 S Warren and O Gates, Cancers induced in different species by continuous γ-radiation *Arch Environ Health* 17 (1968) 697–704
59 R D Evans, On the carcinogenicity of inhaled radon decay products in man (CORD) In *Radiation Exposure of Uranium Miners Hearings before Subcommittee on Research Development and Radiation on the Carcinogenicity of Inhaled Radon Decay Products in Man* Part 2, pp 1188–1207, 1967
60 J F Park, W J Bair, W J Clarke and E B Howard, Chronic effects of inhaled plutonium dioxide in dogs In, USAEC Report BNWL-480, pp 57–61 Pacific Northwest Laboratory, Richland, Wash , 1967
61 E P Radford, V R Hunt, and J B Little, Carcinogenicity of tobacco smoke constituents *Science,* 165 (1969) 312–313
62 E L Wynder and D Hoffmann, Experimental tobacco carcinogenesis *Science* 162 (1968) 862–871

DISCUSSION

P. E. Morrow: Would you hazard a guess as to what the cumulative rad dose would be in those plutonium dogs at the 15-year level, at your so-called threshold, in other words?

W. J. Bair: We can calculate doses ranging from 2500–20,000 rads These would be based on the assumption that the radiation energy is uniformly absorbed throughout the lung

N. Nelson: Dr Bair, as usual, has given an excellent review of this problem However, I do disagree with his statement that the production of experimental lung cancer from radiation is "neither easy nor predictable " On the contrary, I would say the production of lung cancer is both easy and predictable This has been shown in several instances The data that you showed, Bill, which were from the work of Kuschner and Laskin, do show that the dose response curves are highly reproducible over the range of a few percent up to essentially 100% The other point I would like to make relates to your implication that tumors were produced only at "high" doses and not at "low." I don't know what "high" or "low" is, but certainly in our work tumors were produced at local dose levels of the order of a 1000 rads, in this case a 1000 rem I would regard this as low, although not the lowest dose that is capable of producing tumors, but of more concern, it is really the size of the (dose) of that order of magnitude I am sure you could use the same 1 or 2%, which, when used with any reasonable RBE, brings the dosage within the range to which the miners

were exposed. Thus I would say that we are dealing with dose ranges that are highly relevant to practical circumstances of exposure.

The third point I would make is that I would not overemphasize the failure of the radon daughter exposures to yield tumors in experimental animals. This merely illustrates the frequent failure of simulation experiments to faithfully simulate actual exposure.

Bair: I don't disagree at all with what you have said. I would be more accurate in emphasizing that it is extremely difficult to produce pulmonary cancer in animals by inhalation exposure.

M. G. Hanna, Jr.: I would like to raise a point about the data which showed the radiation-induced lung tumors in the different strains of animals. It may be that here is an example of what might be interpreted as a synergistic effect between an infectious agent and radiation. Rats have the highest incidence and the hamsters the lowest incidence of chronic respiratory infections. In most commercial rat colonies, a source undoubtedly used for these experiments, the rats carry a chronic respiratory virus and undergo chronic respiratory infections. As far as I know, the hamsters are very resistant to these. I don't know that we really have been able to detect respiratory virus in hamsters by systematic monitoring. The higher level of cell proliferation, as a result of these respiratory infections in the rat lung, might be a contributive factor to the enhanced radiation-induced lung carcinogenesis.

Bair: I agree. I think this is highly probable.

J. B. Little: I would like to mention some work that Mrs. Grossman and I have been doing in our laboratory in regard to the induction of lung tumors by ^{210}Po alpha radiation. We have been administering ^{210}Po adsorbed onto hematite particles by intratracheal injection, using a technique similar to that described by Saffiotti. These are preliminary results from experiments that are currently in progress. In one group we have given Syrian golden hamsters a series of weekly intratracheal injections of 0.2 of μCi of ^{210}Po adsorbed onto hematite particles and suspended in saline.

We are now in the 40th week of this experiment. The first tumor appeared 15 weeks after the beginning of the series of injections, and by the 40th week 82% of the animals that died had either single or multiple bronchogenic carcinoma. These are all mixed adeno and squamous cell carcinomas of the lung.

So the hamster exposed by intratracheal injection of ^{210}Po adsorbed onto hematite particles indeed seems to be relatively sensitive to the induction of bronchogenic cancer by alpha radiation, with tumors occurring early and in a high percentage of the animals.

Bair: That's very interesting. We are also doing rather extensive studies with hamsters and are happy to know of studies in which the susceptibility of hamsters to radiation-induced pulmonary neoplasia is being demonstrated.

L. D. Marinelli: I have two remarks to make. The first is concerned with dose. I think that talking of dose averages in these circumstances, when the dose-rate changes vary rapidly from point to point by ranges of 10^5, is a little misleading. And I have a suggestion to make in that respect, namely, that probably the statement of rad-grams, which is the product of the rad delivered times something proportional to the number of cells at risk, would be much more meaningful than just talking about average rads.

There is another thing that I would like to mention, and that is the statistics that have been coming out of the epidemiological studies involving humans burdened by several grams of thorium dioxide colloidal particles in the system. In them the active gas, thoron, is exhaled through the lung. There have been several studies on that, and all agree that practically 10 to 12% of the thoron produced in the body is exhaled. Since in a very short time the thoron decays to a considerable degree in the lungs, the respiratory tree is irradiated by it and by its daughters, of which the longest half-life is $10\frac{1}{2}$ hr.

The statistics that come from both Portugal and Denmark, and to some extent also from Sweden, demonstrate that the incidence of lung cancer in these cases is practically nil. Women in Spain, for instance, don't show any lung cancer at all in the 500 cases which were followed for about 25 years. But I think the cases in Denmark do show five instances of lung cancer out of about the same number of men, mostly smokers. In these cases the cancers have appeared at times that are not related to the time of administration of thorium dioxide; they seem to be due to the smoke more than to anything else.

I think it ought to be kept in mind that these cases have certainly not been in uranium or other mines, and I think the hypothesis of synergism in the latter is supported by these data.

E. P. Radford: Much of the discussion of this paper has centered around dosimetry problems, and I'm afraid these are going to be with us a long time. Marinelli's comments are quite relevant here, because if thoron and its daughters, which have very short half-lives, are present at some point in the bronchial tree, the only way that the radiation can affect the basal cells is by penetration through the entire thickness of the mucous sheet.

If you take the data that Dr. Nelson showed this morning, the equivalence between working level months and rad dose that is being used in New York is quite different from the one being used in Hanford, by approximately a factor of 3. We've done our own calculations and we can come up with some numbers, too, but the point is that the dose equivalent ranges anywhere from 1 to 7, and may be even less than 1 rad per working level month.

Now the difficulty here, as I see it, is that we don't have agreement on precisely what model we are using. Perhaps the speaker or Dr. Nelson will comment.

Bair: I tried to point out in my paper that this is exactly the case. I don't think we are at the point where we can identify the correct dosimetry model for

inhaled radionuclides In most cases we haven't identified the target tissue yet, and until we do, I think it's meaningless to express radiation dose except in some way such as Dr Marinelli suggests

Nelson I would reply to Dr Radford by saying that an agreement by a factor of 3 in these estimates is mighty, mighty good!

What we are dealing with here are estimates secured by highly contrived and circuitous routes, and we are basing them on a series of assumptions which are interlocking and interdependent, and we do not have all the parameters to reinforce the several steps We must then have recourse to plausible assumptions

Nevertheless, this does give us a hold on the magnitude of the problem I don't really think that this indirect approach is going to be the way to resolve the problem It is only an interim solution The better answer is going to come from really carefully designed simulation experiments, and again I can't overemphasize my own feeling that poorly contrived simulation experiments are misleading, that they throw-up clouds of confusion which take years to dispell

One final comment is that I do feel that we know what the target tissue is I think we know very well that it is basal epithelium, and that I think that dosage estimates for this tissue are erroneous and again misleading In short, I don't think there is much utility in expressing total lung dosage of radionuclides that have a penetration depth of the order of 50 to 100 μ

Thus we need to define the dosage to the target tissue and the susceptible cells, in highly precise terms

M. Kuschner I have a historical comment, to start with Part of the recent legend of carcinogenesis has been that the "Bergkrankheit," the disease of the miners in the Erzgebirge in 1556 when Agricola described it, was indeed lung cancer I don't think we know that at all

As a matter of fact, Max Pinner, in his lovely little book on adult tuberculosis, uses this disease as an example of the presence of silicotuberculosis in these miners, and he mentions the fact that many of the women had five husbands, because they had all died of this disease I don't think they died of lung cancer!

Now the importance of this, I suppose, is that if it wasn't lung cancer perhaps cigarette smoking becomes more important again People have tried very hard to find some way in which the miners in the 15th century were exposed to hydrocarbons, and in Agricola's book there is a picture of the kind of fires they lit to crack the rocks, since they didn't have other way of doing it However, it may be that it didn't become lung cancer until the turn of the century

I think the issue of contributory damage comparable to that induced by cigarette smoking must be emphasized in all of the experimental models that I'm familiar with This includes the earliest, Lisco's cerium experiment Lisco made the point that tumors arose in areas of preceding damage derived from retained radionuclides

I think this was true, too, of the tumors that your group described, Dr Bair And of course with our pellets we injure the bronchi as we put these in We do induce another kind of injury to the bronchus which may well simulate cigarette smoking.

The issue of infection that was mentioned by Dr Hanna is important in our experiment, too. These rats developed obstructive pneumonia behind and around the pellet

I think this issue applies to Herman Cember's relatively nontraumatic intratracheal installation as well. His animals in the areas of involvement had peribronchial fibrosis and a fair amount of squamous metaplasia before the tumors developed.

MODELS FOR THE STUDY OF PARTICLE RETENTION AND ELIMINATION IN THE LUNG

7 21 1400

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ABSTRACT

The direct or inferred relationship between appropriate measurements of aerosols or gases and inhalation risk has been the basis for most of environmental hygiene and health physics practice in the assessment of airborne hazards A modern extension of this concept is the design and application of lung models for the purpose of developing more realistic evaluation procedures, which, moreover, can utilize variations in environmental conditions to affect changes in risk estimations Examples are the several tracheobronchial models expressly designed for radon daughter dosimetry and the Lung Dynamics Task Group (ICRP) model proposed for radioactive aerosols generally. — This presentation briefly reviews particulate deposition information as an introduction to the main topic, the elimination of nongaseous materials from the respiratory system. The models discussed are (a) the amended ICRP Committee 2 lung model, (b) a new kinetic description for tracheobronchial clearance, and (c) a new conceptual basis for generalizing particulate elimination from the lung parenchyma. The mechanisms and pathways underlying these models provide cogent information for understanding lung responses to environmental contaminants even when simple dose-effect relationships are not available or applicable

The deposition of airborne particles in the lungs is the outcome of the intrinsic instability of aerosols and the spatial and temporal relationships which develop between the respiratory structures and aerosol particles during respiration. Graphic representations of these particle size interactions are available from several sources,[1] an example is given in Fig 1 This size-deposition relationship depicts what can be termed average particle deposition probabilities for a man breathing spontaneously under sedentary conditions. It is a composite made from many experimental studies with monodisperse aerosols[2-5] and from theoretical predictions.[6-8]

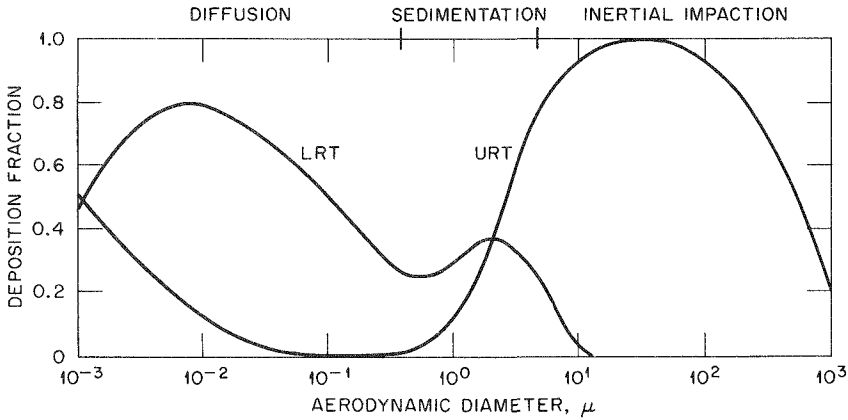

Fig. 1 – Particle size-deposition probabilities for particles from nearly molecular dimensions to those in the visible range. The deposition probabilities for the submicronic size range is theoretical and unconformed experimentally. The two respiratory divisions, URT and LRT, denote upper and lower respiratory tracts, respectively; URT is equivalent to the supraglottic airways, whereas LRT is the tracheobronchial tree and parenchyma. The factors causing deposition are indicated in general terms: there is no sharp demarcation between them, and for many particle sizes all three are effective. The term *aerodynamic diameter* is used to describe the behavior of an unit density sphere in air (*i.e.*, in terms of its terminal velocity of motion). A particle exhibiting the same aerodynamic mobility is assigned that aerodynamic size regardless of its actual geometric size and shape.

Some newer experimental data, relative to that used for the curves in Fig. 1, are available from Lippmann and Albert[9] and Hursh and Mercer;[10] these data were used to construct a similar relationship (Fig. 2). Although precise agreement for all respiratory regions and particle sizes is lacking, it can be seen that much of the data in Figs. 1 and 2 agree satisfactorily. Such particle size-deposition relationships can obviously be utilized in risk evaluations of dust exposure, since they can provide useful models for intake or dose estimations. In addition, they are useful in understanding dust removal processes, although it appears that the size of the deposited particle at a given anatomical level of the respiratory system may not be as significant as the fact that the clearance process varies at different anatomical levels. On the basis of available evidence, it is fruitful to relate the pattern of deposition to the pattern of clearance in general terms. There is evidence that particle size affects specific clearance mechanisms (*e.g.*, phagocytosis, solubility, *etc.*), but so far this information has resisted quantification and generalization. A clearance model which could relate to the parameters of the aerosol breathed would be highly desirable.

The first clearance model to be discussed is one based on an earlier version described at the Haverford Symposium on Airway Dynamics,[11] and is pertinent to tracheobronchial clearance. The information on which this model is based

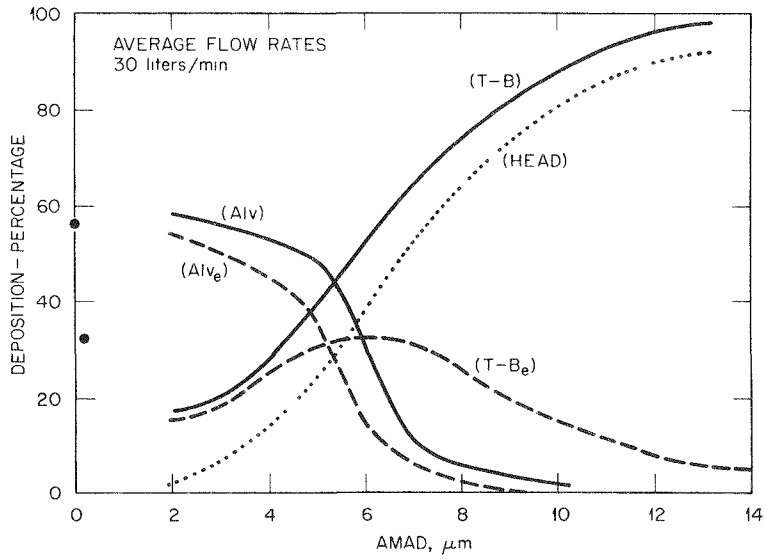

Fig. 2 – Recent data on deposition in the human respiratory tract The symbols have the following meaning T-B (solid line) describes the theoretical deposition efficiencies for the tracheobronchial region, T-B_e (dashed line) denotes the effective deposition curve for the supraglottic airways because of prior deposition in the HEAD, but principally the oral pharynx, Alv is a deposition curve for the alveolar region expressed in terms of particles entering the trachea, Alv_e is the mean alveolar deposition curve based on the inhaled aerosol These data are based on average inspiratory flow rates of 30 liters/min and are taken from ref 9 The two mean deposition values of 33 and 56% are from Hursh and Mercer's studies with 0 20 and 0 02 μm diameter particles, respectively [10]

comes from individuals briefly exposed to an "insoluble" aerosol labeled with a γ-emitting isotope and the deposited radioactivity measured by an array of external detectors placed symmetrically about the thorax. In such an individual, the deposited material often seems to follow a simple power function of the type

$$R_p = At^{-n} \tag{1}$$

where R_p is the fractional retention, A equals the retention value at time 1, t is time, and n an exponent The change in tracheobronchial clearance induced by particle size can be graphically demonstrated by comparing the values of n (rates of clearance) against the median sizes of the particles breathed See Table 1 and Fig. 3.

Note in Table 1 that the value of A decreases and the value of n increases as the median particle size increases The product of An is not found to be a constant, however, but averages 0.13 with a range of 0.08 to 1.6, it is possible to replace the values of A and n with $1.7/D_m$ and $0.07D_m$, respectively, where D_m

TABLE 1

Representative Values for Power Function Model of Early Clearance

($R_p = At^{-n}$)

AMAD* (μm)	A	n
2	.85(.74–.96)	.08(.04–.11)
3	.65(.42–.75)	.20(.16–.30)
4	.52(.25–.70)	.30(.16–.46)
6	.40(.18–.66)	.40(.30–0.80)
8	.33(.18–.48)	.50(.40–0.90)
10	.26(.14–.39)	.60(.50–0.90)
15	.10(.05–.35)	1.00(.70–1.50)

*Activity median aerodynamic diameter.

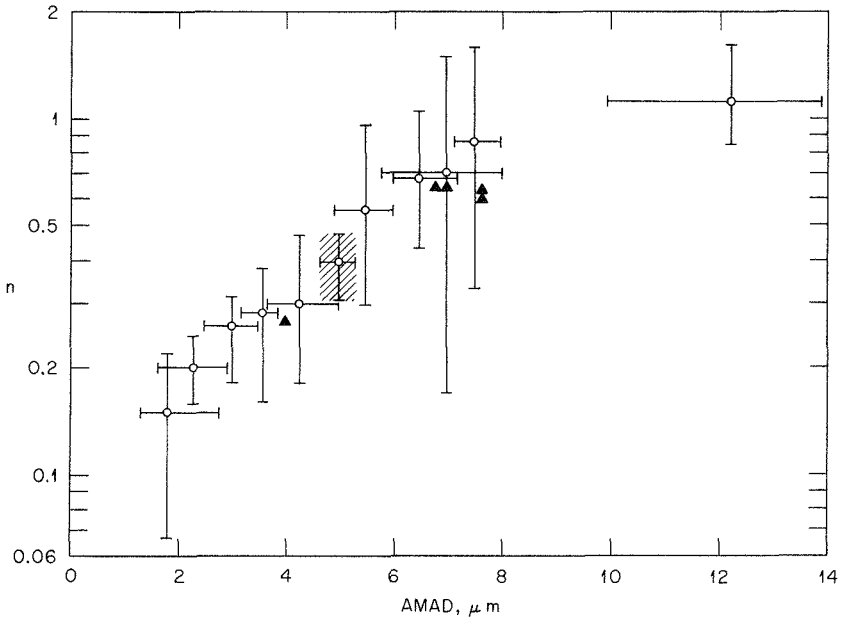

Fig. 3 – Pooled deposition data from many studies with monodisperse aerosols (see ref. 11) analyzed by a power function model. The n, or slope, values from the resulting equations are plotted as a function of the AMAD of the inhaled aerosols. The mean values are depicted by open circles; the horizontal and vertical bars refer to the range of aerosol sizes pooled together and the scatter in the n values for the limited size range, respectively. The dark triangles depict the mean results of eleven studies with heterodisperse aerosols. The cross-hatched area at 5 μm represents the mean results from the investigation of Thomson and Short[12] on normal subjects.

is the activity median aerodynamic diameter (AMAD). Naturally, these representative values are based on data which have considerable variability, as clearly revealed in Fig. 3. It can be seen here that different particle size studies were pooled and represented by a single average value. An AMAD of 2, for example, actually pertains to studies covering a median size range from 1.3 to 2.5 μ diameter. The measured clearance curves from many different studies have similarly been averaged for average particle sizes. The variability in clearance data cannot be completely explained, but there were important technical differences among the various studies (*e.g.*, differences in exposure duration, differences in time between the beginning of the exposure and completion of the first count, *etc.*). This latter factor alone is likely to produce important errors in clearance measurements – especially with the larger particles, which experience a very rapid clearance.

Heterodisperse aerosols, expressed by their AMAD, also seem to fit the foregoing scheme (Fig. 3), but the number of studies available is quite limited. In those studies depicted, the geometric standard deviations (σ_g) of the aerosols varied between 1.5 and 2.2.

Considering all of the studies utilized in Fig. 3, the aerosols concerned had widely differing physical and chemical properties, but were more or less similar in being highly "insoluble" and "physiologically inert." It is evident, therefore, that the tracheobronchial clearance mechanism is best considered a physiological one, in which particle size mainly affects the distribution of particles, and can thereby reveal a gradient of clearance velocities associated with mucociliary transport at different levels of the tracheobronchial tree.

The foregoing view is consistent with regional clearance measurements[11] which indicate a transport gradient from the mediastinal area to the lateral aspect of the thorax. That is, the trachea and upper bronchial airways are initially clearing at approximately 1 cm/min, whereas the more peripheral airways have initial rates of approximately 0.01 cm/min. By utilizing a series exponential model,

$$R_s = f_1 e^{-\lambda_1 t} + f_2 e^{-\lambda_2 t} + f_3 e^{-\lambda_3 t}, \tag{2}$$

a reasonable fit can be made for most human experimental data when the values of λ_1, λ_2, and λ_3 are 1.4, 0.28, and 0.14, respectively; the time is expressed in hours; f_1, f_2, f_3 are intercept values; and R is the fractional retention.

For small particles, the early triphasic clearance appears to run its course in a few hours; the subsequent slow clearance observed is believed to be governed by and characteristic of the lung parenchyma.[13] As will be discussed later, parenchymal clearance is different for different kinds of aerosol materials, but if we restrict our attention to polystyrene, ferric oxide, and other materials used for human clearance studies, it is safe to assign to the slow clearance phase a λ_4 value $\leqslant 0.001$. Thus, to completely describe the first 12 to 24 hr of lower respiratory tract clearance for AMAD's of less than about 8 μ, it is necessary to

add a fourth exponential to the series equation, although it pertains to pulmonary clearance and not tracheobronchial clearance. A group of suitable f and λ values for a series exponential model of tracheobronchial clearance is given in Table 2 Note that for AMAD's greater than 10 μ, no appreciable parenchymal clearance occurs and for particles of 15 μ or more, no more than two exponents are necessary to describe 95% or more of the cleared material

TABLE 2

Representative Values for Series Exponential Model of Early Clearance

$(R_S = f_1 e^{-\lambda_1 t} + f_2 e^{-\lambda_2 t} + f_3 e^{-\lambda_3 t} + f_4 e^{-\lambda_4 t})$*

AMAD† (μm)	f_1	f_2	f_3	f_4
2	05	05	25	65
3	15	20	25	40
4	25	30	20	25
6	45	25	15	15
8	58	25	10	07
10	67	20	08	05
15	85	10	05	

*$\lambda_1 = 1\ 40, \lambda_2 = 0\ 28, \lambda_3 = 0\ 14, \lambda_4 = 0\ 001$

†Activity median aerodynamic diameter

Returning to the power function model (Table 1 and Fig 3), these data plotted on semilogarithmic paper give a group of curves that closely approximate the Table 2 data plotted in the same way (see Fig 4) The Table 2 compilation demonstrates that the physiological functions λ_1, λ_2, and λ_3 can be considered constants, whereas the values of f, the compartmental fractions, change according to the particle size Actually the values of f and λ selected for a given study are subject to the radioisotope and the counting array employed (*i e*, the energy of the radiation, the size and number detectors, the type of collimation, *etc*) The counting array for regional measurements usually consists of a group of individual detectors (1 to 8) or a gamma camera positioned at the anterior or posterior aspect of the thorax, but not both The field of measurement, as with the symmetrical array, always involves a heterogenity of respiratory structures, that is the nature of the lungs Consequently, interpretations and the kinetic values assigned are greatly affected by the experimental design. Also, it is obvious that one can empirically approximate power functions of the types encountered in early particle clearance by the use of the series exponential, but the Table 2 values for $\lambda_1 - \lambda_3$ are better than arbitrarily derived constants in that they can be interpreted in terms of effective mucociliary transport

Several tracheobronchial clearance models have been proposed by Altshuler *et al*,[14] Jacobi,[15] Thomas,[16] Haque and Collinson,[17] and others These models

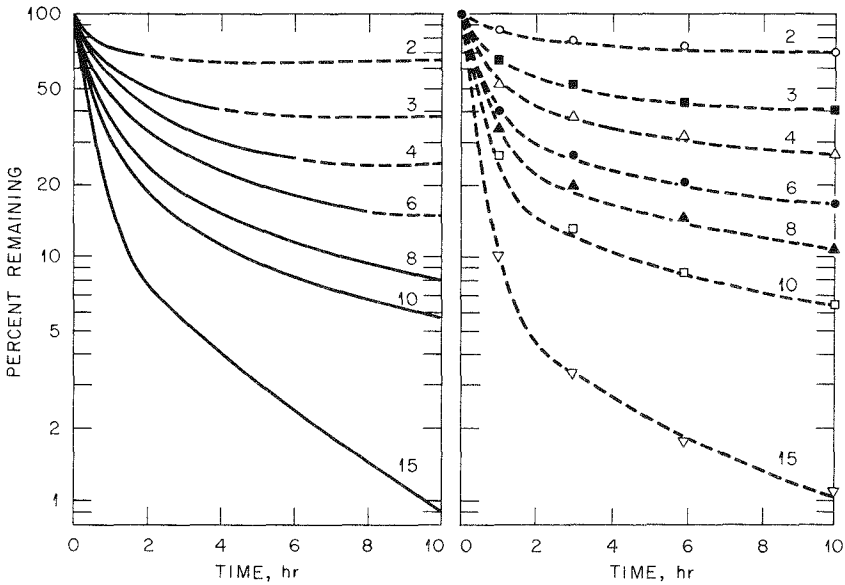

Fig. 4 Left Curves constructed from the Table 2 values. Those portions of the curves associated with parenchymal clearance (f_4, λ_4) are indicated by the dashed lines. Right· Curves constructed from the Table 1 data. The arbitrary points at 1, 3, 6 and 10 hr were computed and connected by dashed lines.

were designed for the specific problem of the inhalation of the progeny of radon-222 and include deposition concepts of Landahl[18] and the general method of calculation of Shapiro.[19] Experimental clearance data relevant to the bronchial tree are a vital part of their approach. However, since these are basically dosimetric models for short-lived alpha emitters, they require dimensions for the tracheobronchial tree, the mucous layer, and the bronchial epithelium at various bronchial levels. It is not useful to review these models in detail, but their dependence upon morphometric and cytologic information is of great importance. These aspects of the dosimetric model for radon progeny can be generalized; relationships between different effects and radiation dose, *etc.*, are totally lacking or are at best, estimates.

The lung parenchyma is clearly a more complex area with respect to both deposition and clearance phenomena. We have already referred to some alveolar deposition data, and it is useful to recall that most of the data is based upon theoretical models, serial histologic and autoradiographic examinations, and washout data or inferences from clearance information (*i.e.*, from that described as the "slow" or alveolar phase). Obviously, these methods of determining alveolar deposition are both indirect and presumptive. Unfortunately, and in the absence of a new approach, more reliable quantitative information cannot be expected.

One of the most interesting models that has been created for dust retention in the lung parenchyma is from Mercer[20] He designed his model with the concept that a log-normal distribution would be retained in the lung Davies,[21] Einbrodt and Amt,[22] and others[23,24] have measured the size of particles in the human lung residues and have found them generally to be log-normal Mercer's approach depends further upon the dissolution of the distribution in a nonequilibrium manner at a rate proportional to the surface area of particles involved On this basis, he found that the rate at which the mass of the deposited particles dissolved is a function of the parameters of distribution, that is, the mass median diameter and σ_g, and a dimensionless parameter β, which in turn is proportional to the solubility rate constant for the material and the mass median diameter of the initial distribution At a given value of β, the ratio of mass remaining (M) to the initial mass (M_0) can be approximated by a biphasic exponential

$$\frac{M}{M_0} = f_1 e^{-\lambda_1 \beta} + f_2 e^{-\lambda_2 \beta} \tag{3}$$

where f and λ are functions of σ, and σ the log of the geometric standard deviation, σ_g The biphasic exponential is useful for distributions with σ's between 0 5 and 1 0 (σ_g 1 5 – 2 7) At larger values of σ, it may be necessary to include a third term in the exponential expression to describe the mass disappearance On the other hand, when single exposures are considered, the standard deviation of the distribution is not an important factor in the dissolution kinetics, provided that the value of β is less than 1, another useful fact is that β has nearly a constant value of 0 6 at $M/M_0 = 0\ 5$, regardless of the distribution Thus, in terms of prolonged lung clearance, Mercer's model predicts that the initial half-time expressed in days will be equal to

$$0\ 6 \frac{\alpha v\, \rho}{\alpha s\, k} D_m \tag{4}$$

In the foregoing expression $\alpha v/\alpha s$* can be assumed to be constant for a dust with a value of approximately 0 1, ρ is equal to the density of the material in g/cm^3, D_m is the mass median diameter in centimeters, and k is the solubility constant for the material From this expression it is apparent that if the value of k is of the order of 10^{-7} g/cm^2/day and D_m is of the order of 10^{-4} centimeters (1 μ), then the half-time of the initial long-term component will be approximately 10^2 days

If the *in vivo* solubility or dissolution rate of a material is the dominant feature of parenchymal clearance, and there is a substantial amount of evidence supporting this viewpoint,[24] then it follows that the "inert, insoluble" materials

*$\alpha v/\alpha s$ denotes a ratio of volume and surface shape factors

being used for human work should be expected to have extraordinarily long alveolar retention times. Unfortunately, solubility data (k values) are quite limited, and as a consequence tests of Mercer's model have been severely hindered. It should be stressed that the solubility value required is the nonequilibrium rate of dissolution of a material in a medium with the approximate composition of lung fluid. It is safe to say that this type of information is greatly lacking and will only be obtained by people in the lung clearance field who are interested enough to approach the problem through Mercer's model.

Work in our laboratory over the past decade has revealed that much of the lung clearance information obtained experimentally in dogs and human subjects fits well into the picture of a solubility model. The test systems we have employed[25] can be considered under two general headings. The first involves the removal rate of sterile intramuscular injections of the aerosol material from an injection site and the second an ultrafiltration technique in which the aerosol material was studied in a medium of the same general composition as lung fluid. The injection test often provided the same type of kinetic information as was obtained from lung measurements in man and experimental animals for the same material; this certainly suggests that a dissolution mechanism is more prominent than endocytosis. The ultrafiltration test has also proven very useful in providing a ranking of materials according to their retention tendencies (*i.e.*, the greater the tendency to persist in the alveolar region, the lower the ultrafiltrability). Both of these techniques are empirical in character and do not generate constants which can be used to characterize the behavior of materials in the lung rigorously; they are effective in providing relative retention information and they tend to support the solubility concept as a dominant removal mechanism for the parenchyma.

The final model to be discussed is that of the ICRP Committee 2, which was first proposed by the Lung Dynamics Task Group several years ago.[26] This model was designed especially for radiation dosimetry of the respiratory system but it should be clear that it can be generalized to other toxicological applications. For practical reasons, it is a less realistic model than some of those we have already discussed, particularly in regard to tracheobronchial clearance. The general respiratory model is portrayed in Figs. 5 and 6.

The model makes use of fixed clearance coefficients for the tracheobronchial tree and the nasal passages, and has a variable pulmonary clearance component, depending upon the chemical nature of the material. Total and regional deposition are predicted in terms of the parametric functions of the inhaled aerosol, *viz.*, AMAD and σ_g. Thus, the model is an amalgamation of many of the concepts which have been discussed in the foregoing models, but it obviously lacks the sophistication and detail which is possible.

For dosimetric applications, the model is readily transformed into mathematical expressions. Figure 5 is used to determine, from sampling information, D_3, D_4 and D_5, which are the amounts of radionuclide deposited in the N-P,

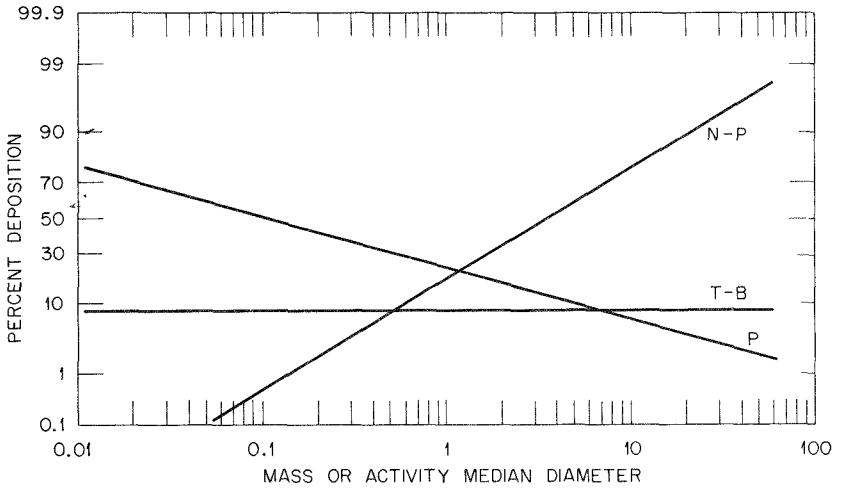

Fig. 5 – A provisional rendition of the deposition model proposed by the ICRP. The N-P line denotes the total mass or activity which will be deposited in the nasal passages as a function of various aerosol distributions. The aerosol distributions are characterized by the single parameter, the mass or activity median aerodynamic diameter. The T-B and P line indicates the depositions predicted for the various distributions in the tracheobronchial tree and pulmonary (parenchymal) regions, respectively. For example, if the air concentration was 100 pCi/liter, and the AMAD was 5 μ, the model indicates that 60, 8, and 10 pCi will be deposited in the N-P, T-B and P regions, respectively, for each liter of air breathed.

T-B, and P regions, respectively. The pathways involved and the fractional amounts, f_i, and clearance half-times, T_i are specified in Fig. 6. Since all of the clearance processes are assumed to be exponential, all of the mathematical formulations for dose are similar. For example, the residence time for dust in the N-P region is determined by clearance pathways (a) and (b); therefore, the accumulated μCi days are

$$D_3\left[\frac{f_a}{\lambda_a + \lambda_r} + \frac{f_b}{\lambda_b + \lambda_r}\right] \tag{5}$$

where λ_a equals $\ln 2/T_a$ and λ_b equals $\ln 2/T_b$, λ_r equals the decay constant of the radionuclide, and T_a and T_b are the clearance half-times for the N-P region pathways. For the T-B region the pattern is somewhat more complex, in that the deposited material, D_4, is resident in the tracheobronchial tree as follows:

$$D_4\left[\frac{f_c}{\lambda_c + \lambda_r} + \frac{f_d}{\lambda_d + \lambda_r}\right] \mu\text{Ci days}. \tag{6}$$

CLEARANCE MODEL

COMPARTMENT		CLASS D $T_{1/2}$ da	D f	W $T_{1/2}$ da	W f	Y $T_{1/2}$ da	Y f
N-P (D_3 = 025)	a	001	05	001	01	001	001
	b	001	05	001	09	001	099
T-B (D_4 = 008)	c	001	095	001	05	001	005
	d	02	005	02	05	02	095
P (D_5 = 025)	e	05	08	50	015	500	005
	f	na	na	10	04	10*	04
	g	na	na	50	04	500*	04
	h	05	02	50	005	500	015
L	i	05	1.0	50	10	500	01

*MEAN TRANSIT TIME IN T-B, 03 DAYS

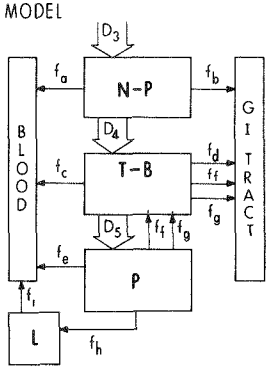

Fig. 6 – Clearance model for human respiratory system. A scheme is developed whereby various materials whose retention time in the P compartment is determined by pathways e, g, and h can be grouped into three broad classes depending upon whether their clearance half-times will be relatively brief (hours to days) intermediate (weeks to month) or prolonged (months to years). These classes are termed D, W, and Y, respectively. With this determination made, the appropriate model is used for which all clearance pathways are given two values, a clearance fraction f and a biological half-time, T/2. For additional details consult ref. 26.

However, some of the material deposited in the P region is removed via the T-B region, and this is given as

$$D_5 \left[\frac{f_f \lambda_f}{\lambda_f + \lambda_r} + \frac{f_g \lambda_g}{\lambda_g + \lambda_r} \right] + \frac{1}{\rho + \lambda_r} \mu\text{Ci days} \tag{7}$$

where, as before, the various subscripts denote the clearance pathways involved and ρ equals the mean passage time material from the parenchyma to the tracheobronchial tree, and this is assumed to be 3 hr. It is evident from the calculation of the material entering T-B from P that this is determined by processes f and g.

There are many publications which contain mathematical formulations for implementing the Committee 2 model into practical dosimetric expressions.[27-29]

The tracheobronchial dosimetric models described earlier indicated some special concern for the relationship between deposition pattern and the presumed structure at risk, the basal cell layer of the bronchial tree. The ICRP model has a general policy of averaging dose over the entire lower respiratory tract (*i.e.*, T-B and P regions) except when the radiation is very weak or from an alpha emitter. In these cases, the mass of the respective respiratory regions is utilized in the dose calculation.

This policy clearly neglects the nonuniformity of deposition which is commonly found with all types of aerosol exposures Dealing with the problem of the nonhomogeneity of the radionuclide burden and lung structures is formidable and nearly impossible to generalize, to the extent that it can cope with the reality of the problem Nevertheless, several interesting approaches bearing on this matter and that of lung cancer have been reported One by Dean and Langham[30] considers the possible tumorigenicity of particles of different size within the lung alveoli, a second by Bevan and Haque[31] speculates upon the possible size of the malignant foci attributable to inhaled alpha emitters, and a third by Sanders *et al*[32] attempts an analysis of carcinogenic risk from nuclear rocket engines

Ultimately, and particularly where lung models are applied to estimates of tumor or cancer risk, such features as microdosimetry, quality factors, cell turnover, co-factors, etc , will probably be integrated into the picture This of course presumes that simple dose-effect relationships for lung cancer will not be forthcoming

ACKNOWLEDGEMENTS

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REFERENCES

1 T F Hatch and P Gross, *Pulmonary Deposition and Retention of Inhaled Aerosols,* Academic Press, New York, 1964
2 D C F Muir and C N Davies, The deposition of 0 5 μ diameter aerosols in the lungs of man *Ann. Occup Hyg*, 10 (1967) 161–174
3 A M Van Wijk and H S Patterson, The percentage of particles of different sizes removed from dust laden air by breathing *J Ind Hyg Toxicol* 22 (1940) 31
4 H D Landahl and R G Herrmann, On the retention of airborne particulates in the human lung *J Ind Hyg Toxicol.* 30 (1948) 181–188
5 I B Wilson and V K LaMer, Retention of aerosol particles in the human respiratory tract as a function of particle radius *J Ind Hyg*, 30 (1948) 265
6 W Findeisen, Uber das Abstzen kleiner, in der Luft suspendierte Teilchen in der menschlichen Lunge bei der Atmung *Pfuger s Arch Ges Physiol*, 236 (1935) 367
7 H D Landahl, Particle removal by the respiratory system. Note on the removal of air-borne particulates by the human respiratory tract with particular reference to the role of diffusion *Bull Math Biophys* 25 (1963) 29
8 J M Beeckmans The deposition of aerosols in the respiratory tract I Mathematical analysis and comparison with experimental data *Can. J Physiol Pharmacol*, 43 (1965) 157–172
9 M Lippman and R Albert, The effect of particle size on the regional deposition of inhaled aerosols in the Human Respiratory Tract *Am Ind Hyg Assoc J* 30 (1969) 257–275
10 J B Hursh and T T Mercer, Measurement of ^{212}Pb loss rate from human lungs *J Appl Physiol*, accepted for publication 1969
11 P E Morrow, Dynamics of dust removal from the lower airways Measurements and

interpretations based upon radioactive aerosols In, *Proceedings of Airway Dynamics Symposium, Haverford College, August 1968.* Charles Thomas, Springfield, Ill., 1969, in press.

12. M. L. Thomson, and M. D. Short, Mucociliary function in health, chronic obstructive airway disease, and asbestosis. *J. Appl. Physiol.*, 26 (1969) 535–539.
13. P. E. Morrow, F. R. Gibb, and L. Johnson, Clearance of insoluble dust from the lower respiratory tract. *Health Phys.*, 10 (1964) 543.
14. B. Altshuler, N. Nelson, and M. Kuschner, Estimation of lung tissue dose from the inhalation of radon and daughters. *Health Phys.*, 10 (1964) 1137–1161.
15. W Jacobi, The dose to the human respiratory tract by inhalation of short-lived 222-Rn and 220-Rn decay products. *Health Phys.*, 10 (1964) 1163–1174.
16. J Thomas, A method for calculation of the absorbed dose to epithelium of the respiratory tract after inhalation of daughter products of radon. *Ann. Occup. Hyg.*, 7 (1964) 271–284.
17. A. K M. M Haque and A. J. L. Collinson, Radiation dose to the respiratory system due to radon and its daughter products. *Health Phys.*, 13 (1967) 431–443.
18. H. D. Landahl, Removal of air-borne droplets by the human respiratory tract The Lung. *Bull. Math. Biophys.*, 12 (1950) 43–56.
19. J. Shapiro, Radiation dosage from breathing radon and its daughter products. *Arch. Ind. Health*, 14 (1956) 169–177.
20. T. T. Mercer, On the role of particle size in the dissolution of lung burdens. *Health Phys.*, 13 (1967) 1211–1221.
21. C. N. Davies, A comparison between inhaled dust and the dust recovered from human lungs. *Health Phys.*, 10 (1964) 1029–1034.
22. H. J. Embrodt and U. Amt, Die Korngrossenverteilung in Lungestäuben von Bewohnern aus Gebieten und ohne Luftverschmutzung. *Archiv. Hyg. u. Bakterol.*, 153 (1969) 98–104.
23. L. Ulrich and A. Juck, Der Charakter der Staub deposition in der Lunge. In, *Forchr. der Staublungerforsch*, Band 2, pp. 585–592. Niederrheinische Druckerei GmbH, Dinslaken, 1967.
24. J. Cartwright, Airborne dust in coal mines· The particle-size-selection characteristics of the lung and the desirable characteristics of dust-sampling instruments. In, *Inhaled Particles and Vapours*, II, pp. 393–408. Pergamon, 1967.
25. P. E. Morrow, F. R. Gibb, H. Davies, and M. Fisher, Dust removal from the lung parenchyma. *Toxicol. Appl. Pharmacol.*, 12 (1968) 372–396.
26. D. V. Bates, B. R. Fish, T. F. Hatch, T. T. Mercer, and P. E. Morrow, Deposition and retention models for internal dosimetry of the human respiratory tract. *Health Phys.*, 12 (1966) 173.
27. P. J. Dionne and B. O. Stuart, Plutonium inhalation model simulates the long-term burdens of the deep lung and systemic organs. IEEE Region Six Conference, Portland, Oregon, May 1968. C_f Report BNWL-SA-683 (1966)
28. B. Shleien, A. G. Friend, and H. A. Thomas, Jr , A method for the estimation of the respiratory deposition of airborne materials. *Health Phys*, 13 (1967) 513–517.
29. P. Kotrappa, Calculation of the burden and dose to the respiratory tract from continuous inhalation of a radioactive aerosol. *Health Phys.*, 17 (1969) 429–432.
30. P. N. Dean and W. H. Langham, Tumorigenicity of small highly radioactive particles. *Health Phys.*, 16 (1969) 79–84.
31. J. S. Bevan and A. K. M. M. Haque, Some speculation on the carcinogenic effect of inhaled alpha-active material. *Phys. Med. Biol.*, 13 (1968) 105–112
32. C. L. Sanders, R. C. Thompson, and W. J Bair, Carcinogenesis in the Lung from Inhalation of Radioactive Particles with Special Reference to Hazards of Nuclear Rocket Engines, Battelle Northwest Research Report, May 1969.

DISCUSSION

W. J. Bair: Your slide [Fig. 4 in text] showing the family of curves suggested that 1-μ particles would not be cleared. Is this correct?

P. E. Morrow: I don't have any information on 1-μ particles. The number I think you are worried about is in the power function representation where $1.7/D_m \approx A$. A is virtually 100% for 2-μ particles. There must be some slight clearance in the first hour, but this is the way it comes out of the analyses I have done.

The slopes of the clearance curves for the AMAD's of 1.6- to 2.5-μ particles are extremely flat. In the paper I have given the A values and their ranges, which are rather large, as well as the n-value ranges [shown in Fig. 3]. Thus, for this particular situation, A's range between 1.0 and about 0.92, or something of that general order. But my general equation would not be applicable to smaller AMAD's.

M. Lippmann: Although we recognize that the ICRP needed mathematical models for the calculation of lung dose estimates, the fact remains that bronchial clearance doesn't fit either exponentials of power functions, or any other simple mathematical form. What actually takes place in most cases is a series of steps. There may be no clearance for intermediate periods for as long as several hours during the first day, when bronchial clearance is taking place. Also, lung retention doesn't approach an asymptote, but rather ends suddenly when the most distal mucus, at the time of deposition, has reached the larynx.

I mention this point because we have at New York University data from over 200 human inhalation tests, a much larger number than that from all the other investigators I know of put together. So while you can fit a mathematical relation to the data, it just isn't that way at all.

Morrow: That's a very interesting interpretation because I'm using much of the data from NYU, and Dr. Lippmann knows as well as I do that models have to generalize and simplify, and they do miss a lot. On the other hand, breathing a single breath of an aerosol of uniform particle size is not the real world either, but it does tell us something about what goes on.

Lippmann: These are not single breaths. The inhalations last from one to several minutes.

Morrow: That makes it very complicated because the absolute time of the exposure, the absolute time of the measurements, and the absolute times all the way through are mandatory with this type of analysis. Yet this kind of information is not always available. Often it has to be surmised from the experimental description.

Lippmann: In our work, which has been described in the open literature, the times have been stated: usually a 1-min period of inhalation, with the first measurement 1 min later.

Morrow: That's what I've used with your data.

Lippmann: The fact is that on this time scale with monodisperse aerosol not depositing uniformly but in discrete areas, it is not surprising that there should be times when nothing is discharging from the trachea. Many times there are discrete waves of clearance, sometimes two, sometimes more; and as I said, I recognize that you can fit mathematical relations to the curves. But this isn't the real world at all.

Morrow: I don't know what you mean by that, because we have to use "fits" in all of physiology. There are always enormous individual variations and averages, and simplifications are justified.

Lippmann: It's a gross misreading of the experimental data when 15-μ and 10-μ particles are still clearing at 10 hr after the end of inhalation.

Morrow: Did you see what the percentage was?

Lippmann: Yes, but it's wrong.

Morrow: For the sake of a graphic representation, it's given as below 1%. Now, can you measure 1%?

Lippmann: It was in the case of 10 μ. It wasn't in the case of 15.

Morrow: I don't remember the values exactly. With 10-μ particles, you would expect to have about 18% left after 1 hr; with 15-μ AMAD's it would be 10%. So it's an extremely rapid clearance and faster for 15 μ than 10 μ. The 10-hr value for 15-μ AMAD was less than 1%.

The clearance of 10- and 15-μ aerosols can be described rather well by a single exponential, occasionally two. Often they are poor fits to a power function by the same token, because it's only when you have a multiphasic exponential that you can utilize the two types of analyses interchangeably. Large particles are also undergoing the fastest removal. So the amount you have at the end of the very first measurement is crucial. Variations in experimental design cause this to be quite variable. If we discuss clearance after nasal breathing, then we would probably have to change the whole picture, because most of these experiments which I have utilized are based upon mouth breathing. My idea of bringing forth this analysis was merely to show the possibility of dealing with rather complex phenomena, which are highly variable in man, and the possibility of tying it together with the parameters that can be measured in the inspired air. I think these possibilities exist. I would not want anyone to take these particular curves seriously because most of them were made with mouth breathing, and I don't think it's valid to put these data against the model at the present time.

Lippmann: I don't want to pursue this any further, but I would like one point of information from you. You mention a revised ICRP Committee II Task

Group Model. Has it been published? Is it available in any form? I presume this model supersedes the one that was published in *Health Physics* several years ago.

Morrow: Yes, that's right. The amended Task Group Model was adopted by Committee II this past spring and will be published, hopefully, by next winter! It has been in preparation for six or eight months.

R. Montesano: I wonder if you had some information concerning the deposition and the clearance of particles of the same size after intratracheal instillation and inhalation.

Morrow: I really know very little about particle clearance after intratracheal injection, but you might anticipate that particles will not be deposited or cleared as a function of their aerodynamic characteristics. They will be gravitated into areas they might not normally penetrate, but I know of no specific information on the point.

M. Schneiderman: I feel that Lippmann and Morrow are really not so far apart as it might appear. What Lippmann is saying is that the process that is going on is a discrete process; each breath is a discrete breath.

Morrow has said that, "I've looked at some mathematics which assumes that I've got a continuous process going. If it really were a continuous process, it would tell me this." For example, the mathematics makes a gross approximation in saying that the tracheobronchial tree is divided into three parts. It's not. It's a continuity. And to divide it into three parts is artificial. He knows it's artificial. However, the mathematics of handling the continuity would be different from the mathematics he gave, but three compartments as an approximation to continuity may still tell us a lot.

The mathematics of handling the discrete process that you get in the inhale-exhale process is very complicated. An analogy exists from the epidemic theory. There one often starts out by saying that there is a continuous infection process going on, *i.e.*, a continuous addition of susceptibles and continuous removal of susceptibles when they become infected. This is what was being done in the first malaria models. After 20 or 30 years of fruitful experimentation based on what was clearly not the true state of the world, somebody came along who could handle the mathematics, and he made the models more meaningful by trying to incorporate a discrete model.

The mathematics related to stochastic processes, which may allow this, is difficult. The Morrow Model may develop along these lines. I hope people will be able to handle the mathematics when the model is made more realistic.

Thus, I see these two gentlemen as being not far apart. I think Lippmann is pressing Morrow to give him more realistic models, and the pressing is worthwhile.

On the other hand, Morrow's models are much more realistic than the ones that existed earlier. So long as we don't freeze on a model, we'll make progress

and get closer to reality. Thus I see something coming of both the disagreements and the agreements.

Morrow: I wish I had said that! I certainly agree. Lippmann and I have had many discussions about these things, and I, too, believe we are not really so far apart; but if he, or anyone else, is really disturbed by these mental gymnastics, I wish he would try to come up with something better. I don't believe it's all so chaotic.

D. Craig: In reply to the question asked by a previous speaker concerning the particle size versus deposition characteristics following intratracheal injection or inhalation of material, there is a report [J. A. Watson *et al., Arch. Environ. Health,* 19 (1969) 51–58] in which it is demonstrated that very little difference exists between particle size distribution and deposition sites.

E. P. Radford: I would like to bring out a question that Dr. Morrow and I have discussed for many years. Paul, do you think that the compartment consisting of the bronchial epithelium itself is an important compartment to be added to any model that is derived for bronchial clearance?

Morrow: Yes, I think this is the consensus of the ICRP's Committee II. Consequently, for alpha emitters and very weak beta emitters, that is, of low energy (10 kev or less), they advocate the use of the mass of the bronchial tree for the dose calculation instead of the whole lung; whereas, with more energetic photons, they use the whole lung and tracheobronchial mass. That is a concession toward your idea, but it doesn't have the quality, I'm sure, that you would like to see, or that was attempted in the tracheobronchial models which were designed for radon progeny.

K. H. Kilburn: I am tempted to speculate in the following direction. Because the lung in man has approximately 300,000 respiratory units, not all of these units are used at one time. Therefore it is possible that only a portion of these units are entering into the clearance system at any one time, for example, only a portion are entering into ventilation or perfusion. Therefore the trailing off of clearance at the end of the exponential could be due to the fact that units are only participating intermittently in clearance. The implications of Dr. Lippmann's argument may be explained by irregular recruitment and derecruitment of units. Observations of recruiting and derecruiting of ventilatory units during inflation and deflation suggest that their clearance may also be recruited and derecruited. Thus is it suggested that slower clearance from smaller airways may reflect more minutes during which units are not clearing compared to the minutes during which they are clearing. It would be easier for me to understand in terms of information about the mucociliary apparatus rather than that they have fine gradations of their clearance rates.

SESSION I

INHALATION TECHNOLOGY

Chairman – D. G. Doherty
Biology Division
Oak Ridge National Laboratory
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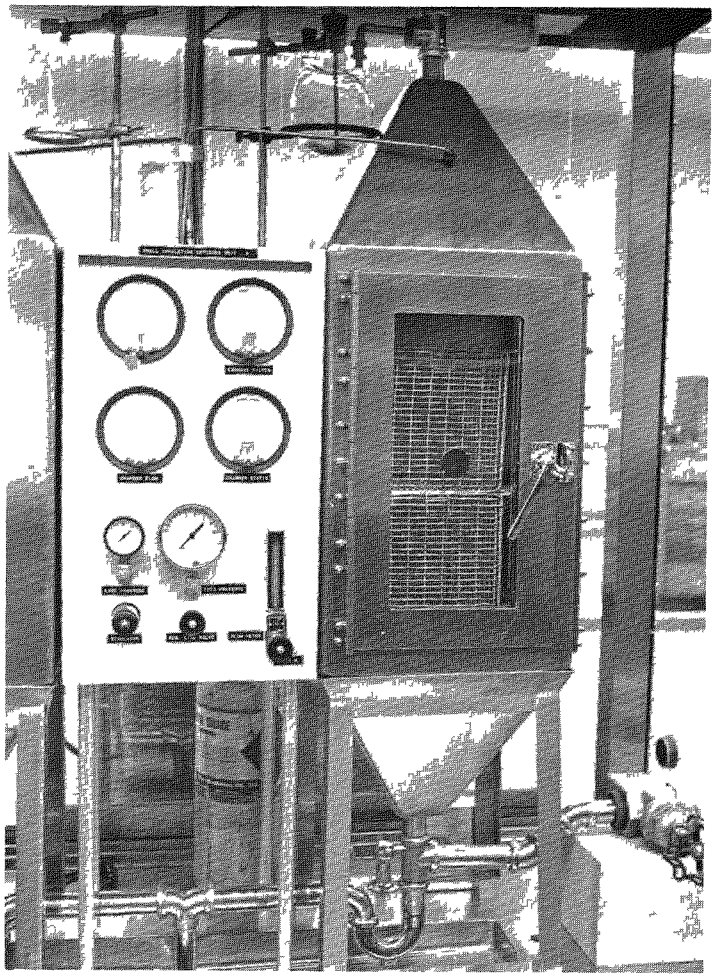

GENERATION AND CHARACTERIZATION OF AEROSOLS

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ABSTRACT

Common techniques for the generation and characterization of aerosols are reviewed, with emphasis upon the experimental nontherapeutic applications The methods of aerosol generation described include dispersion from dry powders and dusts and the atomization of solutions and suspensions with various compressed air and ultrasonic nebulizers Emphasis is placed upon the generation of aerosols of "insoluble" forms under controlled conditions Particular attention is given to the various methods of producing "monodisperse" aerosols from liquids – including spinning disk, electrostatic dispersion, controlled condensation, and ultrasonic dispersion The relationships between generation techniques and the resulting characteristics of the aerosols are summarized The importance of aerosol characterization in the evaluation of inhalation problems, and the instruments and techniques used for such analyses are discussed with respect to the various standard experimental approaches The uses of electrostatic and thermal precipitators for electron microscopic studies, of filter samples for gross analyses, and of various dynamic samplers such as impactors, centripeters, cyclones, elutriators, and aerosol centrifuges are reviewed The various particle "sizes" employed and common size distribution terminology are described with special concern for their relationships to the factors associated with the inhalation of toxic or radioactive materials

Aerosols, relatively stable suspensions of liquid drops or solid particles in a gaseous medium, are encountered in all phases of life In fact, a fundamental property of air appears to be that it contains aerosols Even in thoroughly filtered air, aerosols will spontaneously form from trace gases under the influence of radiant energy [1–6] A great interest exists in aerosols because of their use in medicine, industry, and research, and because of their collection by the respiratory tree

Fundamental to the use or study of aerosols is the ability (a) to generate suitable forms and (b) to ascertain their nature and character Selected aspects of

these two extensive topics are briefly summarized herein from the perspective of the investigator who is interested in aerosol behavior or the biological effects of aerosols inhaled by animals or man.

GENERATION OF AEROSOLS

General

The extensive use of aerosols in medical therapy has led to the design of many types of aerosol generators; these are generally divided into three basic groups: (1) compressed air nebulizers, (2) ultrasonic nebulizers, and (3) dust blowers. In addition to medical nebulizers and dust blowers, other specialized nebulizers, dust blowers, spray nozzles, and aerosol generation devices are widely used in experimental research. Among these specialized instruments are some designed to produce uniform droplets or particles.

Nebulizers

A nebulizer is an atomizer that produces many highly respirable droplets (*i.e.*, less than 10 μ in diameter, as opposed to those from a spray atomizer which may range up to 100 μ or larger). Both compressed air and ultrasonic nebulizers produce aerosols from liquids. Often, the particles of residue remaining after the droplets evaporate form the desired aerosol.

Important factors in describing the operation of nebulizers include (a) the output rate of usable aerosol, (b) the volumetric rate of air (c) the evaporation losses of liquid which are independent of usable aerosol, (d) the droplet size distribution, (e) the volume of liquid required for proper operation, and (f) the maximum unattended operating time. Cognizance of these factors in relation to a particular application determines the choice of a nebulizer.

The output rate of usable aerosol is usually described as volume of liquid at initial formation associated with droplets that leave the generator and may be expressed in μliter/min. The number of droplets from most nebulizers is between 10^6 and 10^7 per cc of air, but since the volumetric rate of air often is a number of liters per minute, the droplet output rate is usually in excess of 10^9/min.

Evaporation losses increase the concentration of the solute in solution or particles suspended in the aerosolized liquid. This concentration change causes a concomitant increase in the sizes of the particles that are formed when the droplets dry. This evaporation occurs both from the surface of the liquid and from the droplets which evaporate slightly and then hit the wall of the nebulizer to be returned to the reservoir. Evaporation is most important in nebulizers with small reservoirs but large volumetric air flows.

Nebulizers produce droplets of many sizes and resultant aerosol particles after evaporation are concomitantly polydisperse.[7] The droplet distributions described for nebulizers are the initial distributions at the instant of formation; droplet evaporation begins immediately even at saturation humidity since the vapor pressure on a curved surface is elevated.[8] The rate of evaporation depends

upon many factors – including surface tension, energy availability, degree of saturation of the air, the solute concentration, the hygroscopicity of the solute,[9-11] the presence of immiscible liquids or evaporation inhibitors,[12] and the size of the droplets (smaller droplets have higher vapor pressures and dry faster[8]).

The distribution of droplets produced by nebulizers has been described satisfactorily in many ways.[7,13-15] Most useful is the assumption that the logarithms of droplet size are normally distributed. This log-normal distribution of sizes allows for simple mathematical transformations[16] and, usually describes droplet volume distributions satisfactorily.[15] The characteristic parameters of a log-normal distribution are the median (or geometric mean) and the geometric standard deviation (σ_g). The median of a distribution of droplet diameters is called the droplet count median diameter (CMD); the median of the mass or volume distribution of the droplets is called either the mass median diameter (MMD) or the volume median diameter (VMD). These are related by:

$$\ell n(MMD) = \ell n(CMD) + 3\ \ell n^2 \sigma_g \qquad (1)$$

in which ℓn refers to the natural logarithm. A representative log-normal distribution is shown in Fig. 1 for a CMD equal to 1 μ and a σ_g equal to 2.

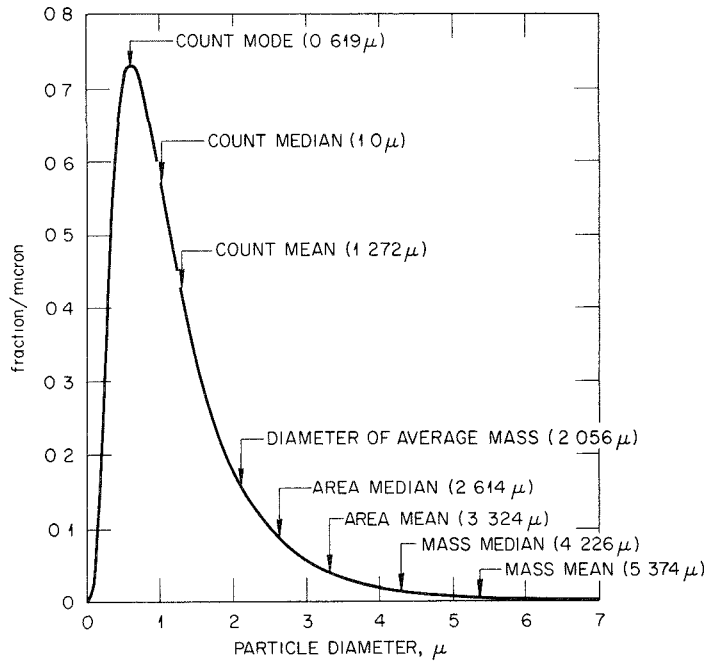

Fig. 1 – An example of the log-normal distribution function in normalized linear form for CMD = 1.0 and σ_g = 2.0, showing the mode, median and mean of the size distribution, the surface area distribution median and mean diameters, the mass distribution median and mean diameters, and the diameter of average mass.

Aerosols produced from aqueous solutions (and some other methods) are charged by the random imbalance of ions in the droplets as they form.[17] After evaporation, aerosol particles can be relatively highly charged; this may cause a small evaporating droplet to break up if the Rayleigh limit[18] is reached due to the repelling forces of the electrostatic charges overcoming the liquid surface tension.[19] The Rayleigh limit, the minimum stable diameter D, of a droplet with n electronic units, e, of charge, can be expressed as:

$$D = \sqrt[3]{\left(\frac{n^2 e^2}{2\pi\tau}\right)} \tag{2}$$

with τ the surface tension. In some cases the net charge on a particle may be tens or even hundreds of electronic charge units, which will affect both the aerosol stability and behavior. Therefore, a reduction in the net charges on aerosols produced by nebulization either by mixing with bipolar ions[20] or by passing through a highly ionized volume near a radioactive source[21] is desirable and, in some experiments, may be imperative.

Compressed air nebulizers generate droplets by shattering a liquid stream with fast-moving air.[22-26] The liquid is usually drawn into the air flow by the natural reduction in pressure that occurs at right angles to the fast-moving air stream (Bernoulli's Theorem). Spray nozzles work by a similar mechanism but may also involve pressure injection of the liquid into the air stream.

The DeVilbiss No. 40 (DeVilbiss Co., Somerset, Pa.) is one of the simplest compressed air nebulizers. This glass nebulizer (Fig. 2) has a vertical jet and a separate capillary through which the liquid to be aerosolized is drawn into the air stream. Its reservoir holds only about 10 ml. About 16 liters of air and 0.22 ml of liquid droplets are released per minute at an operating pressure of 20 psig.

The Dautrebande D-30 nebulizer (Fig. 3) also uses a vertical jet with separate feed capillary.[27] The aerosol droplets, however, are required to follow torturous paths through baffle holes so that most of the larger drops are unable to negotiate this scrubbing action and are collected and returned to the liquid reservoir. Consequently, droplets which leave the Dautrebande generator as useful aerosol are much smaller than those produced by other nebulizers. The D-30 releases about 25 liters of air and 60 μl of liquid droplets per minute at an operating pressure of 20 psig. It is usually constructed of lucite.

The Lauterbach nebulizer[28] uses a jet consisting of a metal tube sealed at one end with a small hole drilled near the sealed end. This jet is operated by attaching a compressed air line to the open end of the tube with the orifice positioned very near the surface of the liquid so that the air stream is emitted parallel to the surface. The liquid touching the tip of the metal tube is drawn directly into the air stream because of the reduction in pressure at right angles to the stream. The Lauterbach model improved nebulization by using a recirculating reservoir system, consisting of the generator reservoir and a larger volume

supply reservoir Liquid from a 200-ml supply reservoir continually flows into the generation reservoir, which is maintained at a constant level with a fixed overflow tube that allows excess liquid to be pumped back to the main reservoir Since the recirculation system works independently of the jet operation, the

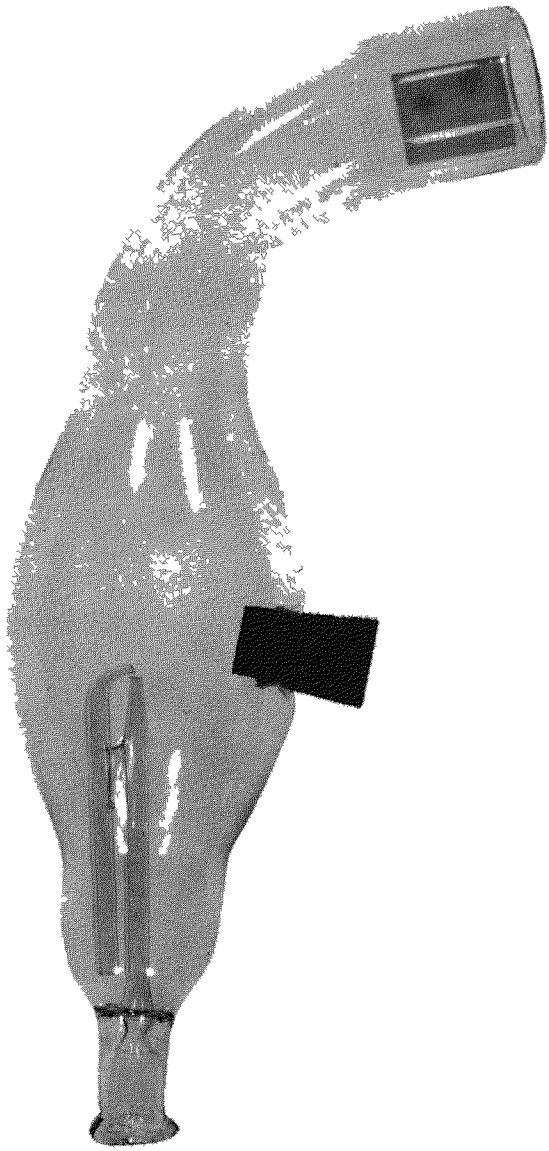

Fig 2 – The DeVilbiss No 40 nebulizer, the compressed air inlet is at the bottom and the vent is corked

liquid is continually mixed and concentration changes are minimized. The reported output is about 2.4 liters of air and 7 μl of liquid droplets per minute at an operating pressure of 20 psig.[28] A schematic of the glass Lauterbach

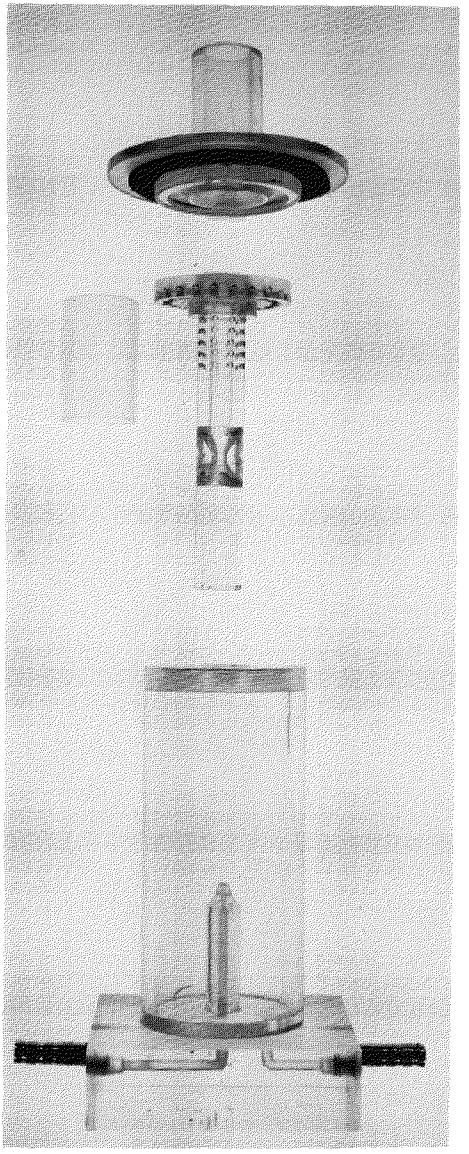

Fig. 3 – Exploded view of a Dautrebande D-30 nebulizer showing the vertical jet, orifice neck, and baffle holes. This unit has a separate liquid feed to allow addition of liquid during operation. (This nebulizer was kindly provided by Mr. Harry J. Ettinger of Los Alamos Scientific Laboratory.)

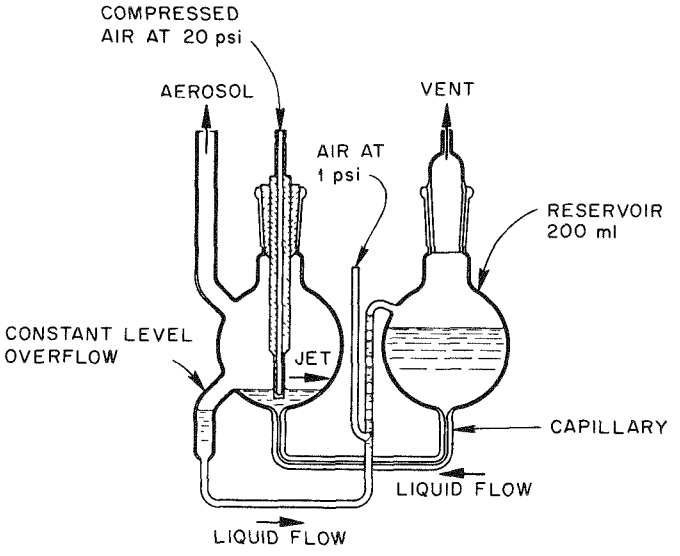

Fig. 4 – Schematic drawing of the Lauterbach nebulizer. (From Lauterbach *et al.*[28])

nebulizer is shown in Fig. 4. Mercer *et al.*[26] report a plastic modification[29] of the Lauterbach generator that is somewhat different in design.

The Lovelace (Fission Product Inhalation Laboratories, Lovelace Foundation for Medical Education and Research, Albuquerque, N.M.) nebulizer (Fig. 5) is a miniature generator which, for its size and simplicity, has an outstanding efficiency in the generation of usable aerosols. It operates with a small liquid volume of about 4 ml and a low total air flow of about 1.6 liters/min at 20 psig jet pressure. This device can provide up to 60 μl/min of liquid droplets. A jet baffle, based upon the principle suggested in a medical nebulizer designed by Wright,[30] provides a second droplet-shattering mechanism which reduces the population of large droplets in favor of smaller ones. Since larger droplets are normally prevented from leaving a nebulizer, the baffle provides a greater output of usable smaller droplets. A small adjustable teflon screw mounted very near and in direct opposition to the air jet serves as this primary baffle in the Lovelace design. Newton *et al.*[31] describe the basic design of the Lovelace nebulizer and indicate among its characteristics (a) relatively high output of usable aerosol, (b) small operating volume (<4 ml), (c) reliable operation for up to 50 min with one loading, and (d) ease of fabrication. The construction is of lucite for the liquid cup, epoxy for the top, brass for the outlet tube, and stainless steel for the tube supplying compressed air to the lucite jet assembly. The jet assembly contains a small orifice (9.2 mil), a cylindrical capillary through which liquid is fed to the jet, and a support for the teflon baffle. Mercer *et al.*[26] stress the importance of positioning the jet baffle, in that there is an optimum

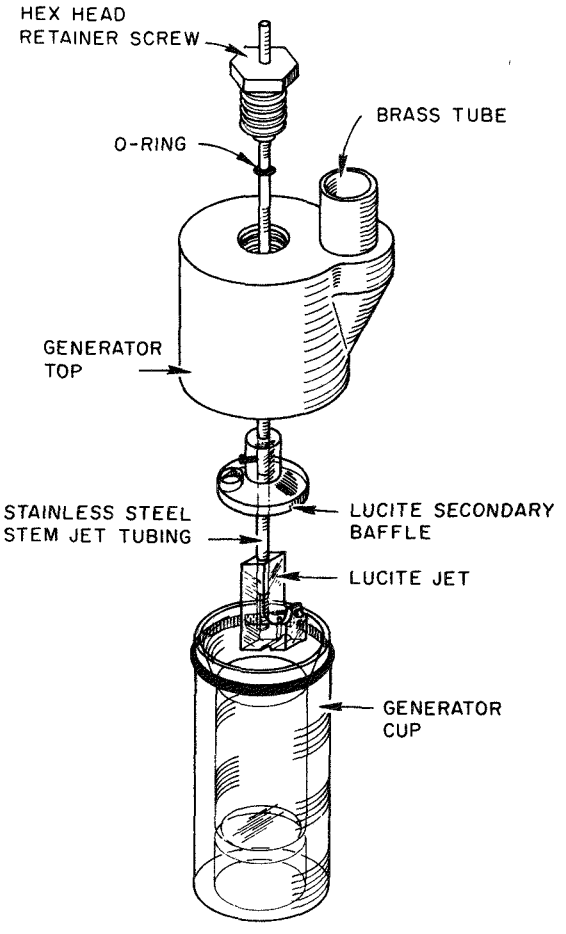

Fig. 5 – Current design of the Lovelace nebulizer.

distance between the end of the screw and the orifice that provides a maximum output of aerosol; they also report a very favorable output-to-evaporation ratio.

The Laskin nozzle (designed by S. Laskin, Dept. of Environmental Medicine, New York University Medical Center, New York, N.Y.) consists of an orifice in a sealed metal tube. The orifice is positioned at the top of a metal capillary through which the liquid is drawn into the air stream. The bottom of the feed tube can be submerged into the liquid at various levels without markedly affecting the aerosol output. Although the efficiency of the Laskin nozzle is not remarkable, it is useful for producing large quantities of aerosol when large volumes of air are either desirable or not prohibitive.

Ultrasonic nebulizers operate differently from compressed air nebulizers.[32-36] Although a flow of air is used to carry off the aerosol droplets, the

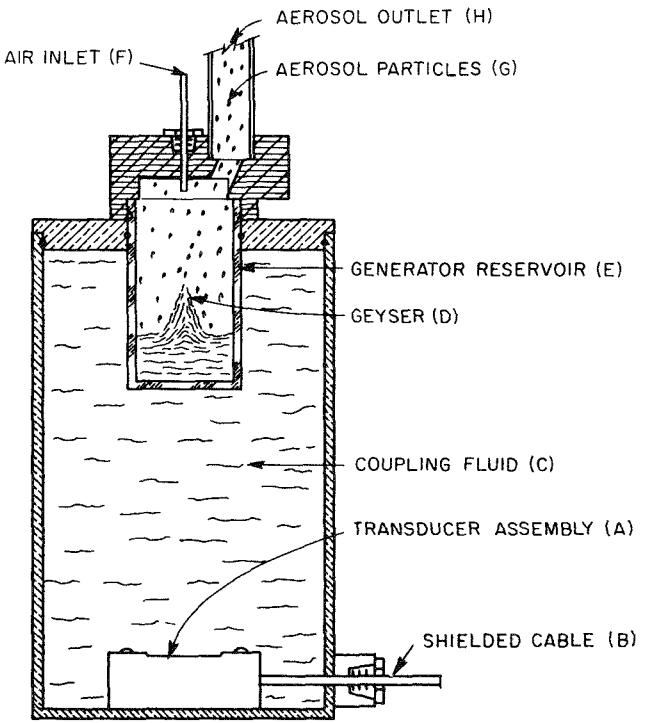

Fig. 6 – Sectional schematic view of an operating Ultrasonic Aerosol Generator showing transducer assembly (A) receiving power through shielded cable (B) generating an acoustic field in the coupling fluid (C) creating an ultrasonic geyser (D) in the generator reservoir (E) and air entering at (F) carrying away aerosol (G) through the aerosol outlet (H). (Figure and description kindly provided by G. J. Newton.)

air is not involved in the initial formation of these droplets. A sectional view of an experimental ultrasonic generator (designed by G. J. Newton, Lovelace Foundation, Albuquerque, N.M.) is shown in Fig. 6. A 110-volt, 60-cycle line current is converted to a high-frequency signal (in this model approximately 800 kHz) and transmitted to the transducer *A* through the shielded cable *B*. The transducer, a cylindrical piezoelectric device, transforms the high-frequency electrical current to mechanical oscillations. Because of the shape and nature of the crystal, these mechanical vibrations are highly directional and create an intense acoustic field in the coupling fluid *C*. The energy is carried through the liquid by the motion of the molecules along the direction of propagation. Intense adiabatic compressions and rarefactions occur with corresponding density and temperature changes. The actual amplitude of the motion is only about 0.2 μ in an 800 kHz field, but the acceleration attained is in excess of 500,000 *g*. This turbulence creates a pressure gradient along the cylindrical axis of the transducer and results in a water geyser *D*. The high-frequency turbulence

TABLE 1

*Representative Characteristics of Selected Compressed-Air and Ultrasonic Nebulizers**

Compressed Air Nebulizers

Air pressure (psig)	DeVilbiss#40 (ref 26) (Jet = 33 mil) (vent closed) Output (μl/liter) (evap)	Total air (liters/min)	VMD (μ) (σ_g)	Lovelace† (Jet = 9 2 mil) Output (μl/liter) (evap)	Total air (liters/min)	VMD (μ) (σ_g)	Dautrebande D 30 (ref 26) (Jet = 41 mil) Output (μl/liter) (evap)	Total air (liters/min)	VMD (μ) (σ_g)	Lauterbach[26] (Jet = 13 mil) Output (μl/liter) (evap)	Total air (liters/min)	VMD (μ) (σ_g)
5	16 0	7 5	4 6 (1 8)	1 6	0 8		1 0 (9 7)	13 4		2 6	1 2‡	
10	16 0	10 8	4 2 (1 8)	15 3 (11)	1 2		1 6 (9 6)	17 9	1 7 (1 7)	3 9	1 7‡	3 8 (2 0)
15	15 5 (8 6)	13 5	3 5 (1 8)	19 5	1 4					5 2	2 1‡	
20	14 0 (7 0)	15 8	3 2 (1 8)	30 0 (10)	1 7	5 8 (1 8)	2 3 (8 6)	25 4	1 4 (1 7)	5 7 [7 2 / 12]‡	2 4‡	2 4 (2 0)
30	12 1 (7 2)	20 5	2 8 (1 8)	26 7	2 2		2 4 (8 2)	32 7	1 3 (1 7)	5 9	3 2‡	2 4 (2 0)

Commercial Ultrasonic Nebulizers[38]

	Output (μl/liter) (evap)	Total air (liters/min)	VMD (μ) (σ_g)		Output (μl/liter) (evap)	Total air (liters/min)	VMD (μ) (σ_g)
DeVilbiss setting #4 (Somerset Pa)	150 (33 1)	41 0	6 9 (1 6)	Mist O_2-Gen with reservoir (Oakland Calif)	61 5 (22 2)	24 7	6 5 (1 4)

*Outputs are given in μl of solution per liter of total aerosols (evaporation losses are in parentheses) Total volume of aerosol is indicated as total air in liters/min The droplet distribution of usable aerosol at initial formation is assumed to be log normal with data given for the volume median diameters (VMD) and geometric standard deviations (σ_g) The sources of the values are indicated by superscript reference numbers or foot notes as appropriate

†Baffle setting has been optimized for operation at 20 psig (The author is indebted to Mr J E Bennick and Mr G J Newton of the Lovelace Foundation for the data on the Lovelace Nebulizer)

‡Authors data

in the geyser produces the aerosol droplets The sizes of the droplets formed depend on the frequency of the acoustical field and the physical-chemical character of the liquid The sizes of the droplets carried by the air stream out of the generator depend upon the rate at which the droplets are carried away from the geyser, since coagulation is rapid at the high concentrations of droplets initially formed For example, with an air flow of 1 liter/min, the droplet distribution had a volume median diameter of 10 μ, while at a flow of 10 liter/min it was only 3 μ [37]

The operating characteristics with water of several compressed air and ultrasonic nebulizers[38] are summarized in Table 1 Only a few operating pressures have been described for each compressed air nebulizer, although they may all be operated at various pressures with concomitant variations in operating characteristics

Commerical "aerosol cans" operate on a very different principle from that of nebulizers They require a mixture of the liquid to be atomized and a volatile liquid (usually a fluoronated hydrocarbon such as dichlorodifluoromethane) The pressure in the sealed can caused by the volatile liquid forces the liquid mixture through a feed tube to the nozzle orifice The rapid evaporation of the volatile liquid upon release from the orifice shatters the liquid stream into droplets which usually have a broad range of sizes, often up to 200 μ in diameter

Production of Aerosol Particles by Nebulization

Aerosols of both "soluble"* and "insoluble"* materials may be produced by nebulization of solutions or suspensions For example, spherical "insoluble" particles of plastics may be made from solutions with suitable organic solvents, or "soluble" forms may be made from aqueous solutions of electrolytes Changes may be made in the chemical state of the particles formed after solvent evaporation by heating, and in the size distribution by selective collection of a portion of the particles

Both crystalline and amorphous forms of aerosol particles may be produced from aqueous solutions Often the type of particles produced will depend upon the conditions of drying as well as the chemical nature of the materials If drying is too rapid, low density particles which are essentially shells may be formed by the encrustation of the surfaces of the drying droplets More often, drying is seriously hindered by the hygroscopicity of the solute,[9-11] the presence of immiscible liquids or evaporation inhibitors,[12] the insufficiency of energy available to the droplet, the saturation of the air, and other factors Adequate drying requires mixing the primary droplet aerosol with clean, dry air Warming of an aerosol to speed drying, by passing it through a heated tube, may be necessary in some experiments

*The terms *soluble* and *insoluble* are relative terms especially when used to describe the character of inhaled aerosols

When droplets evaporate, the residue particles become the aerosol. Since nebulizers produce droplets of many sizes, the aerosols formed after evaporation are polydisperse. The size of a given particle depends upon the solution concentration, δ, the droplet size, D_{drop}, and the particle density, ρ. These are related for spherical particles by:

$$D_{real} = D_{drop} \sqrt[3]{\frac{\delta}{\rho}} \tag{3}$$

with D_{real} the diameter of the resultant particle. Since aerosol particles vary in density and shape, it is useful to relate various size particles in terms of their dynamic characteristics. For example, the aerodynamic equivalent diameter for a particle may be described as the diameter of a unit density sphere with the same falling speed as the particle.[11] The aerodynamic diameters of solid spheres of various densities produced from a 5-μ droplet for various solution concentrations are shown in Fig. 7.

Nebulization of an aqueous colloidal suspension forms insoluble particles of the aggregates of the colloidal micelles. This method has the advantage of requiring no organic solvents. If the micelles are small and in high concentration, the resultant particles will be nearly spherical, and their size distribution may be

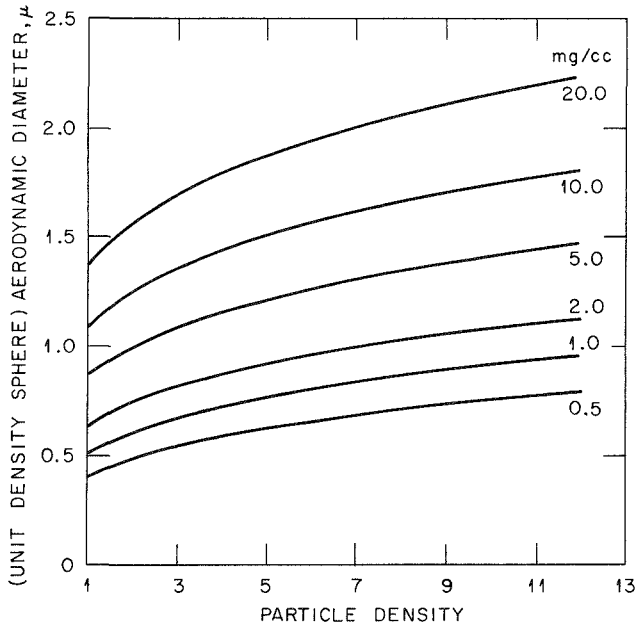

Fig. 7 – The aerodynamic diameters (unit density spheres) for spherical particles of various densities produced by the evaporation of a droplet of 5 μ diameter from various solution concentrations (mg/cc).

predicted by assuming that the suspension behaves as a solution. For example, a colloidal suspension of ferric hydroxide may be aerosolized to produce relatively insoluble and spherical particles of ferric oxide for inhalation experiments.[39] Water physically trapped or chemically bonded in such particles may be removed by heating. If the colloidal micelles are large compared to many of the droplets produced by the nebulizer,[40] or if the concentration is small, physical factors and the statistics of the random pick-up of micelles by droplets will determine the size distribution of the resultant aerosol. Unfortunately, aerosols produced from colloids may have inherent porosity[41] because of the interstices between micellular components. Aerosol of bacteria and viruses can also be produced by nebulization of suitable suspensions.[42]

Changing the chemical nature of aerosols after they are produced is a useful way of creating particles with desirable chemical and physical characteristics. Kanapilly *et al.*[43] describe the generation of spherical particles of insoluble oxides from aqueous solutions with heat treatment of the aerosols. This procedure involves (a) nebulizing a solution of metal ions in chelated form, (b) drying the droplets, (c) passing the aerosol through a high-temperature heating column to produce the spherical oxide particles, and (d) cooling the aerosol with the addition of diluting air. Another example of aerosol alteration is the production of spherical aluminosilicate particles with entrapped radionuclides by heat fusion of clay aerosols.[44] This method involves (a) ion exchange of the desired radionuclide cation into clay in aqueous suspension and washing away of the unexchanged fraction, (b) nebulization of the clay suspension yielding a clay aerosol as shown in Fig. 8, and (c) heat fusion of clay aerosol removing water and forming an aerosol of smooth solid spheres as shown in Fig. 9.

Dry Particle Aerosolization

Dust generators such as the DeVilbiss dust blower (Fig. 10) are designed to turbulently suspend dry dusts and carry the resultant aerosol into an air stream. The DeVilbiss unit uses turbulent air flow in the glass dust container to stir the dust. Many methods may be used to generate dry dust, but all involve two factors: getting the dust in motion by shaking, grinding, stirring, etc., and moving the proper amount into the desired air volume. Some methods may not yield reproducible or unvariable concentrations or size distributions. One unusual approach to dust generation involves the loading of dry powder into a capsule and propelling it with an air gun through a sharp cutting device into an aerosol chamber.[45] This method has even been used for animal exposures.[46]

One of the most popular dust-generating devices has been the Wright Dust Feed,[47] which allows dust to be ground from a packed plug. A timing and gearing mechanism provides a constant rate for this dust feeding device. Another somewhat similar device, providing a narrower size range of particles, has been described by Dimmick.[48]

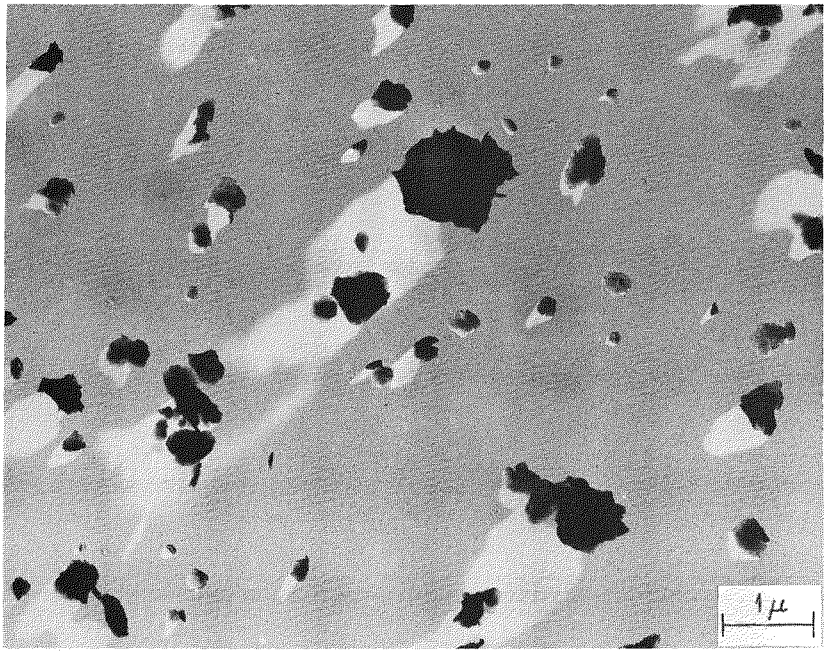

Fig. 8 – Electron micrograph of a sample of clay particles generated by nebulization of a clay suspension.

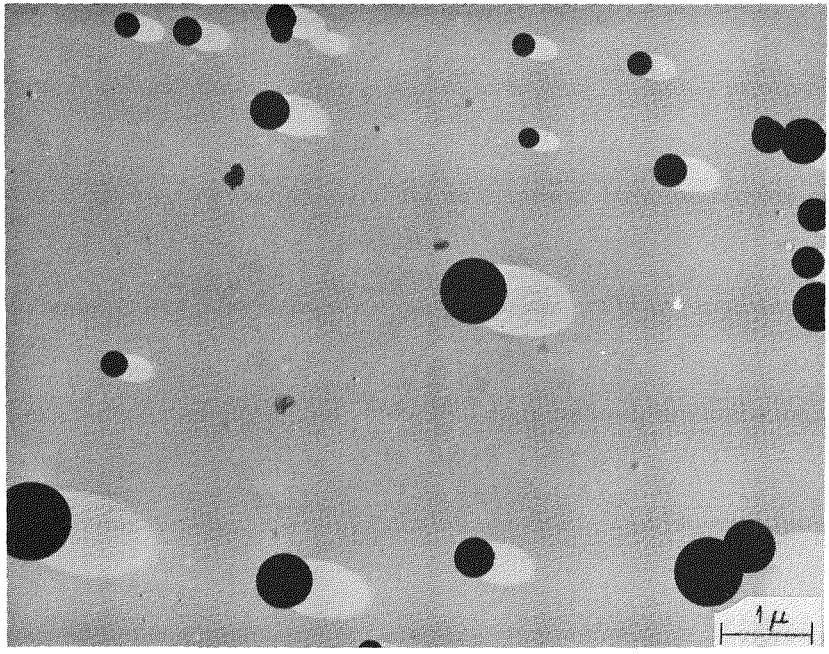

Fig. 9 – Electron micrograph of a sample of fused clay aerosol produced by passing the aerosol shown in Fig. 8 through a heating column.

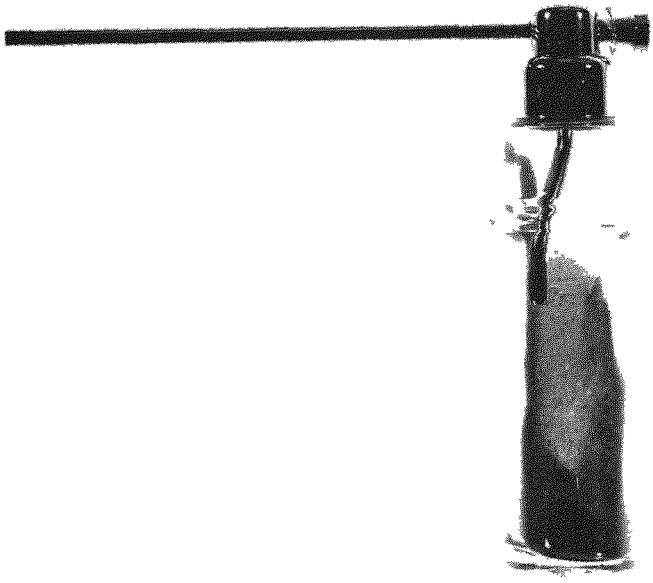

Fig. 10 – The DeVilbiss dry-dust blower used to aerosolize powders.

Useful aerosols of dry particles of metal and metal oxides have also been produced with electrically heated[49] or exploded wires.[50] These techniques have some disadvantages because of the very broad size distributions of the resulting particles and because of the tendency of particles to coalesce. There are applications, however, for this type of aerosol, and it is possible with a technique such as Couchman's[49] wire-heating method to produce spherical particles of many different metals or their oxides. Aerosols of very small particles have also been produced by arc vaporization.[51]

Generation of Monodisperse Aerosols

The generation of aerosols with particles of similar size and physical-chemical characteristics is desirable in some experimental applications. Many of the methods for producing these "monodisperse" aerosols have been reviewed by Fuchs and Sutugin[52] and Liu.[53] The various methods include the growth of uniform aerosol particles or droplets by controlled condensation, and the formation of uniform droplets (which may dry to solid particles) by controlled dispersion of liquids electrostatically; ultrasonically, with a vibrating disperser; or with a

spinning disk or top. Another method for producing monodisperse aerosols is the nebulization of a suspension of monodisperse particles, but it involves some inherent difficulties. It is not easy to produce suitable monodisperse aerosols for particular applications. The variety of physical-chemical types of such aerosols is still somewhat limited and the specialized equipment usually requires careful operation.

Since it is not possible to produce perfectly identical particles, reasonable tolerances must be defined to describe a "monodisperse" aerosol. Fuchs and Sutugin[52] suggest that if the coefficient of variation* of the distribution of sizes is less than 0.2 (20%), the aerosol may be satisfactorily described as having "practical monodispersity." For a log-normal distribution, this is about equivalent to a geometric standard deviation of less than 1.2. These criteria are not very stringent, and most investigators prefer to achieve greater size uniformity. However, even with very effective devices for producing uniform particles or droplets, it is sometimes necessary to tolerate a small fraction of odd-sized particles or doublets (two primary particles which have coalesced).

The nebulization of suspensions of uniform particles, which have been chemically grown, has been a simple and useful means of generating monodisperse aerosols.[54] Suspensions of polystyrene latex spheres of fairly uniform size grown by emulsion polymerization as developed by Bradford and Vanderhoff[55-56] (available from the Dow Chemical Company) have been commonly used for this purpose. Other types of monodisperse hydrosols have also been made.[57-58] Polystyrene latex spheres[59] and others[60] can be labeled with radionuclides. It is not possible at reasonable dilutions to guarantee that only individual particles will be aerosolized and droplets produced that contain more than one of the suspended particles will become undesirable aggregates upon evaporation. Raabe[61] has calculated the dilutions of 10% by volume stock suspensions of monodisperse spheres required to generate 95% single particles; these are shown in Fig. 11 for various log-normal droplet distributions and sphere diameters. Since most of the droplets produced at these low dilutions contain no spheres, they dry to form relatively small particles of residue of the impurities in the liquid; these secondary aerosols can be undesirable.[52,62]

The isothermal growth of droplets under supersaturated conditions by vapor diffusion and condensation causes the droplet surface area to increase linearly with time, as given by Wilson and LaMer:[63]

$$r^2 = r_0^2 + bt \tag{4}$$

with r the droplet radius at the time t, r_0 the initial radius; b may be almost constant under controlled conditions. Hence, if droplets are grown by controlled condensation onto two small nuclei of widely different size, say radii 0.01 μ and

*The coefficient of variation of a distribution is the ratio of the standard deviation to the mean, expressed either as a fraction or a percentage.

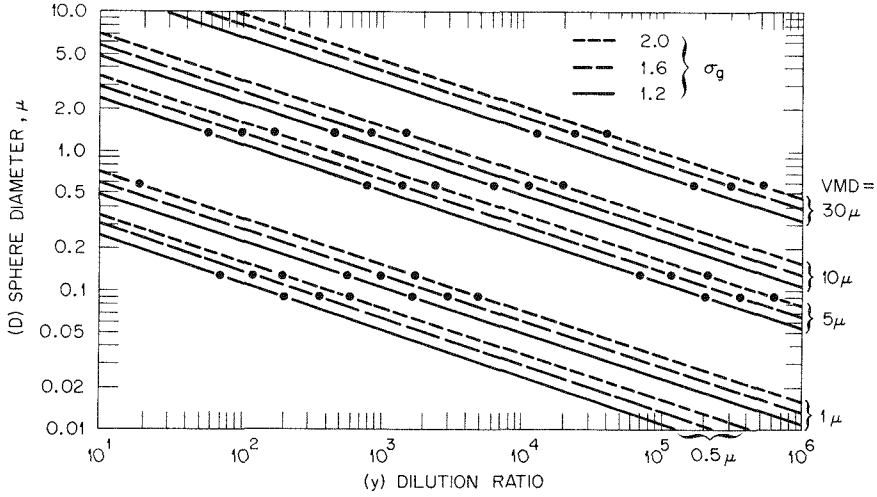

Fig. 11 – The dilution ratio, y, required to generate a singlet ratio (R) equal to 0.95 vs. the sphere diameter from stock of 10% spheres by volume for various values of the volume median diameter (VMD) and geometric standard deviation (σ_g) of the droplet distribution. The lines were derived by an empirical equation and the points calculated numerically by a theoretical equation. (From Raabe[61])

0.1 μ, with bt $\approx 0.16\ \mu^2$ the droplets formed will be almost of identical size with radii of 0.40 μ and 0.41 μ, respectively. Sinclair and LaMer[64] employed this principle to design an apparatus for generating monodisperse droplet aerosols of such materials as oleic acid, stearic acid, lubricating oils, menthol, dibutyl phthalate, dioctyl phthalate, and tri-o-cresyl phosphate. Many researchers have used and improved on the basic Sinclair-LaMer generator,[65-72] and the basic technique has also been used to produce solid aerosols by sublimation.[73,74]

Monodisperse aerosols of both soluble and insoluble forms can be produced from solutions or suspensions if small uniform droplets can be dispersed. These droplets can then be dried and, if desired, altered in character to yield the required aerosols. Many devices have been designed to produce small uniform droplets. Two of these, the vibrating reed[75] and vibrating thread,[76] have created only moderate interest because of the low concentrations available.

Electrostatic dispersion[77] provides another method for generating uniform droplets for certain solutions. Vonnegut and Neubauer[78] and others[79] have studied this approach using a filament of electrically charged liquid released from a small capillary. The filament breaks-up into fragments, forms a conical spray, and further breaks-up into small droplets of almost uniform charge and size. These droplets must be discharged soon after generation.

The most popular device for dispensing uniform droplets has been the spinning disk aerosol generator first described by Walton and Prewett.[80] They observed that primary droplets thrown off at the perimeter of a spinning disk

were of uniform size. Liquid is fed to the center of the disk and flows by centrifugal forces to the edge, where it accumulates until the centrifugal force, which increases with increasing liquid at the edge, overcomes the surface tension and disperses the liquid. This dispersion also produces some secondary fragments (satellite droplets) which are easily separated dynamically from the primary droplets. This is usually done by a separate flow of air near the disk, into which the satellites move but beyond which the larger primary drops are thrown. A spinning disk generator, shown schematically in Fig. 12, can attain disk speeds up to 100,000 rpm. The drop diameter, D, produced by a spinning disk is given theoretically by:[80]

$$D = K\sqrt{\left(\frac{\tau}{\rho\omega^2 d}\right)} \tag{5}$$

with τ the surface tension, ρ the fluid density, d the disk diameter, ω the speed of angular rotation of the disk, and K a constant given theoretically by $\sqrt{12}$,

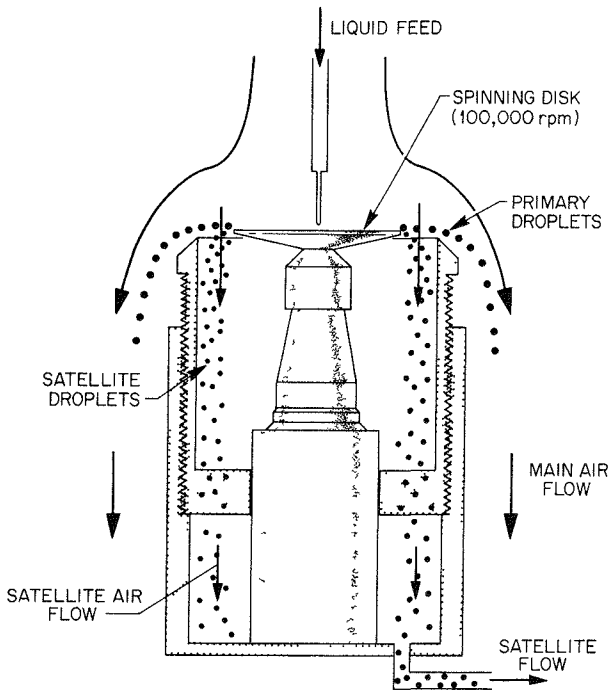

Fig. 12 – Schematic drawing of a Spinning Disk Generator used to produce monodisperse aerosols of both soluble and insoluble forms from solutions or suspensions. Air flow into the satellite collector is adjusted so that the inertia of the primary particles allows them to enter the main air flow.

which varies in practice from 2 to 7. Many investigators have developed and successfully used spinning disk monodisperse aerosol generators for a variety of experimental applications, including aerosol studies[81-87] and inhalation experiments.[88-95]

A new and promising approach to the dispersion of monodisperse droplets is the high frequency ultrasonic disturbance and subsequent disintegration of a thin liquid stream. Fulwyler[96] first described a droplet generator of this type and, later, Fulwyler *et al.*[97] reported its use as a cell separator. A high frequency power signal is converted to mechanical vibrations by an ultrasonic transducer

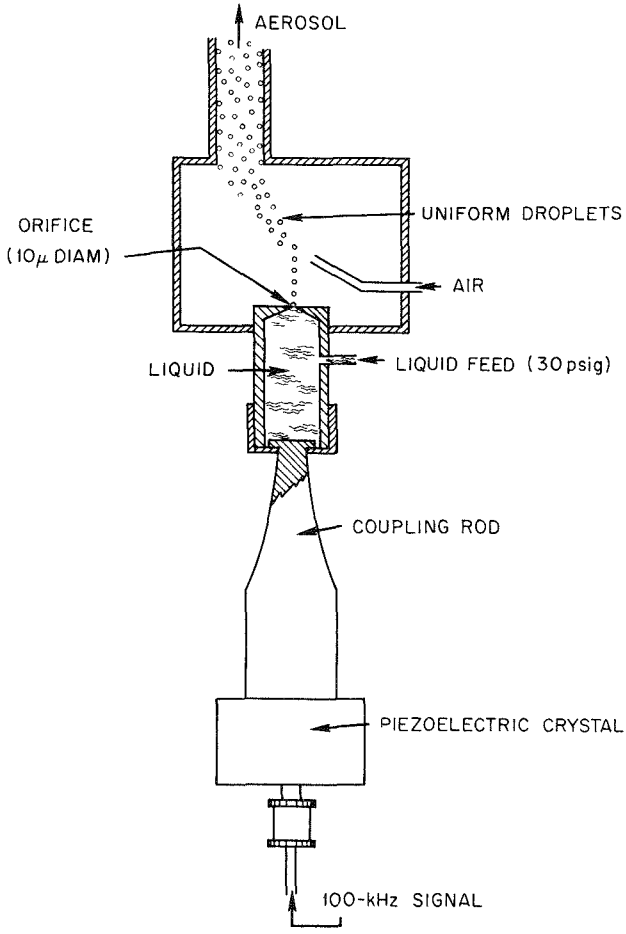

Fig. 13 – Schematic drawing of the Fulwyler Droplet Generator used to produce monodisperse aerosols of both soluble and insoluble materials. Air is directed at the stream of droplets to perturb them out of alignment and create the droplet cloud. (From Fulwyler and Raabe[98])

linked by a coupling rod to a small liquid reservoir. The reservoir is pressurized (≈30 psig) to emit the liquid through a small orifice (≈10 μ) as a fine stream. This stream is uniformly disrupted by the ultrasonic vibrations into droplets that can be made to vary less than 1% in volume. A schematic drawing of the Fulwyler droplet generator for producing aerosols is shown in Fig. 13. Fulwyler and Raabe[98] have produced uniform aerosol particles of insoluble zirconium oxide by dispersing droplets of zirconium oxalate with the Fulwyler generator and heat treating the aerosol by the method of Kanapilly *et al.*[43] An electron micrograph of such an aerosol is shown in Fig. 14. The uniformity of particles

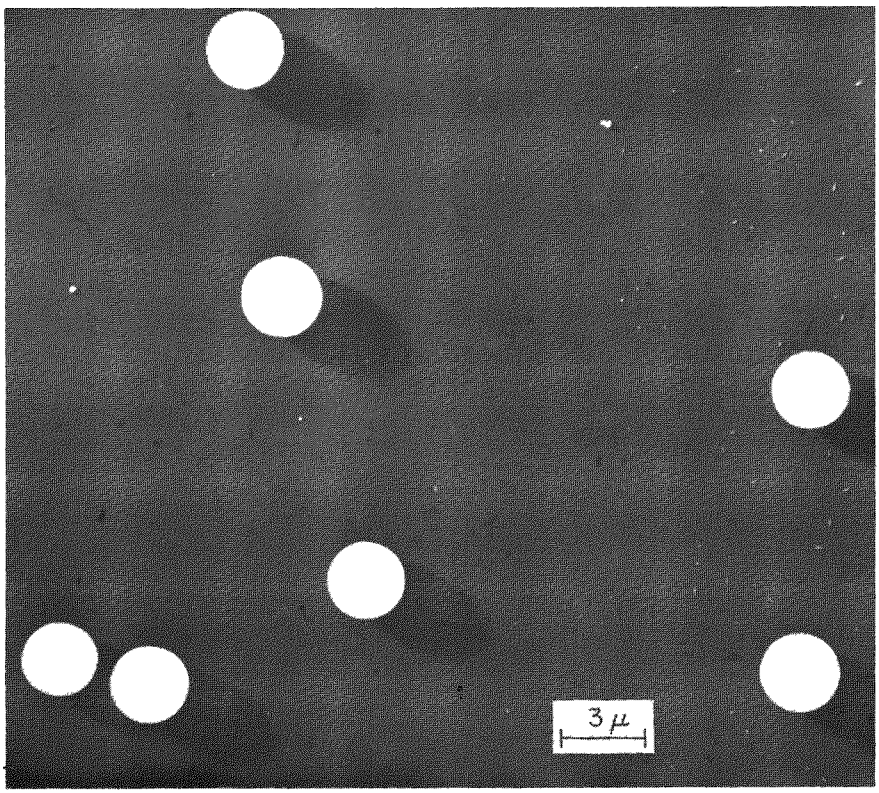

Fig. 14 – Uniform spherical aerosol particles of ZrO_2 produced with the Fulwyler Droplet Generator from solutions of zirconium oxalate and degraded to the oxide with a heating column by the method of Kanapilly *et al.*[43] (From Fulwyler and Raabe[98])

produced with this small (height ≈ 12 cm) and simple device is far better than that previously attainable; the coefficient of variation of sizes of the droplets is much less than 1%. A similar device using electroconstrictive elements around the orifice instead of an ultrasonic transducer has been described by Strom.[99]

CHARACTERIZATION OF AEROSOLS

General

Characterization of an aerosol involves not only determining the size distribution, number concentration, and mass concentration of particles, but also ascertaining information concerning all aspects of their physical and chemical character that may be important in determining the aerosol behavior. These factors may include (a) particle shape, surface area, or volume; (b) particle density; (c) chemical composition and physical state; and (d) distribution of static-electrical charge. Other factors that may be important in certain applications are (a) solubility (as in body fluids in inhalation experiments), (b) radioactive concentration, and (c) aerodynamic character as related to falling speed or diffusion coefficient. There are many devices and methods commonly employed to determine the various aerosol characteristics.

Optical Aerosol Analyzers

Optical observation is the oldest and most basic method for studying aerosols. Specialized techniques and instruments have been constructed which use light scattering and extinction for the direct study of aerosols.[100-117] The Sinclair-Phoenix photometer (Phoenix Precision Instrument Co., Philadelphia, Pa.), for example, uses forward light scattering to estimate aerosol concentrations. The Royco 200 optical counter (Royco Instruments, Inc., Palo Alto, Calif.) uses light scattering to classify particle size groups over the range from 0.3 μ to 10 μ diameter. A simple device for measuring the number concentration of very small particles is the GE Nuclei Counter (Gardner Associates, Inc., Schenectady, N.Y.) which uses light extinction through a cloud of droplets grown upon the small particles under supersaturated conditions. Higher-Order Tyndall Spectra (HOTS), which are created by the scatter of white light from fairly monodisperse aerosols, have been used for particle sizing.[64,118] Studies of aerosols with lasers is one of the newest applications of light-scattering techniques.[119] The theoretical bases of light-scattering techniques have been reviewed by Hodkinson[120] and Van DeHulst.[121] Unfortunately, the Mie and Rayleigh scattering theories complicate definitive size and concentration determinations of unknown submicronic aerosols. Important factors, which may be unknown, include (a) particle shape, (b) particle opacity or refractive index, and (c) effects related to the wavelengths of the light. Most investigators use caution in employing scattering techniques for size-distribution analysis of submicronic aerosols of unknown character.

The use of the light microscope in studying samples of respirable particles[122-123] has given way to the more sophisticated and accurate electron-microscopic techniques.[124-127] Samples suitable for electron-microscopic study may be collected upon thin subtrates on small screens or grids by such means as electrostatic and thermal precipitation.

Filters

The collection of gross samples onto suitable filter media is a useful technique for measuring mass concentration, chemical character, or radioactive content of aerosols. The mechanisms of filtration have been discussed and studied by many investigators.[128-140] At least four processes are involved in filtration: impingement, diffusion, electrostatic precipitation, and sieving.

Samples can be obtained with very high efficiency with membrane filters[141-144] such as those available from Millipore Corporation (Bedford, Mass.) and from Gelman Instrument Co. (Ann Arbor, Mich.); however, air flow through high efficiency filters is limited by their relatively high resistance. Microscopically, membrane filters appear spongelike with external pores that are fairly uniform. They are commercially rated by their average pore diameter in micron units. However, these pore diameters cannot be directly employed to predict their filtration efficiency for a given small particle since they are not sieves and will collect particles that are much smaller than the rated pore size.

Thermal Precipitators

The radiometric effect of a thermal gradient upon aerosol particles causes the particles to move away from hot surfaces and toward cold ones.[145] This phenomenon has been used in the design of thermal precipitators to collect aerosol samples.[146-148] Collection efficiency of particles less than 5 μ in diameter appears to be quite uniform.[149] A simple thermal precipitator is shown schematically in Fig. 15. Aerosols are drawn into the device with a pump and the volume of the sample is measured with a flow meter. Flow rates are usually limited to 50 cc/min or less. A laterally strung nichrome wire is heated electrically to create the temperature gradient that produces a dust-free space. Particles entering the device are collected under the thermal gradient onto either a glass slide for optical study or onto an electron-microscope sample (grid) for electron-microscopic examination.

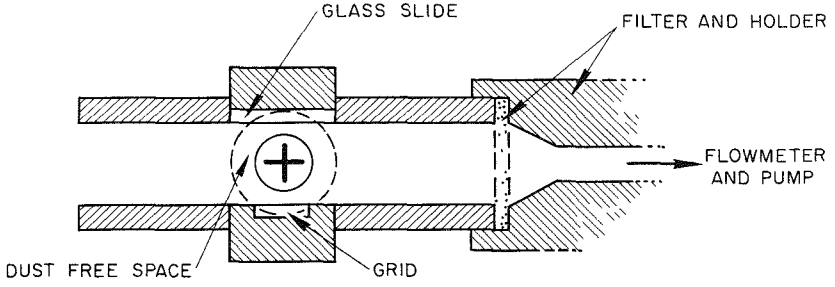

Fig. 15 – Schematic drawing of a thermal precipitator employing a heated nichrome wire (indicated in the drawing by the plus sign) which causes aerosol particles drawn into the device to collect upon either a glass slide or an electron microscope grid for size-distribution analysis.

Electrostatic Samplers

Electrostatic aerosol analyzers are designed to characterize the state of electrostatic charge on aerosol particles. Devices and methods for the analysis and study of the charge with respect to particle size and the electrostatic properties of aerosols have been described by Daniel and Brackett,[150-151] Gillespie and Langstroth[152], Yoshikawa *et al.*,[153] Langer,[154] and others.[155-160] The mobility of charged aerosol particles in various electric fields under a variety of conditions is the primary characteristic upon which these electrostatic analysis methods are based.

Electrostatic precipitators use the forces on charged particles for collecting aerosol samples and for air cleaning.[161] The precipitators provide both the charging mechanism and the collecting field.[162] A concentric configuration, as shown schematically in Fig. 16, has been employed by Barnes,[163] Lauterbach *et al.*,[164] and others.[165] In these concentric electrostatic precipitators, a corona discharge is produced in the converging field lines near a high-voltage electrode which is at the center of a conductive grounded cylinder. Aerosols are drawn into the device and the particles charged and collected upon the inner surface of the tube. A foil liner serves as the collection surface. Gross samples for chemical or mass analysis can be collected in this way and since there is some separation in the deposit based upon mobility, tentative estimates may be made concerning the size distribution of the aerosols. Moderately high flow rates and large samples are possible with this concentric design.

Electrostatic precipitation is also useful in collecting aerosol samples for electron-microscopic examination.[166-168] Morrow and Mercer[166] describe a simple point-to-plane configuration for depositing aerosol samples directly upon a grid. A schematic drawing of the point-to-plane electrostatic precipitator is shown in Fig. 17. A sample is drawn into a cylindrical channel at a chosen flow rate ranging from 50 cc/min to 1 liter/min. A sharp needle near one side of the channel serves as a high voltage electrode and produces a corona discharge. In

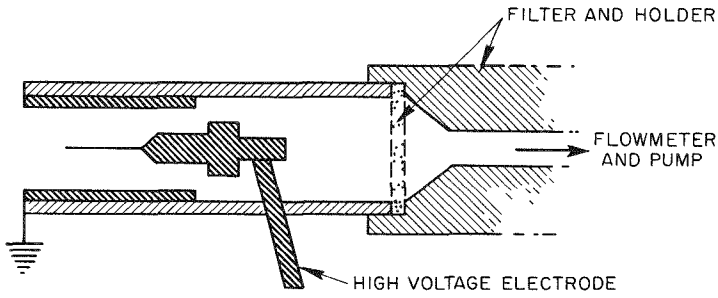

Fig. 16 – Schematic drawing of a concentric electrostatic precipitator which collects aerosols upon a grounded cylindrical tube under the influence of a corona discharge around a high-voltage central electrode.

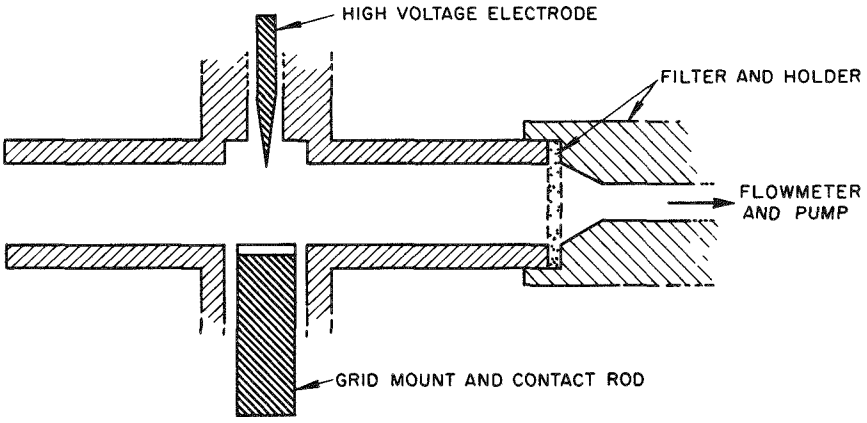

Fig 17 – Schematic drawing of the basic design of a point-to-plane electrostatic precipitator for collecting samples of aerosols onto an electron microscope grid for size-distribution analysis A high-voltage needle supplies ions from a corona discharge, the grid mounting is grounded This drawing illustrates the point-to-plane precipitator designed by Morrow and Mercer [166]

direct opposition to this needle, on the other side of the channel, a carbon-substrated electron microscope grid is mounted on a grounded post Aerosols drawn through this device are charged and collected randomly upon the grid. Mercer *et al* [167] describe a similar instrument (Fig 18) for collecting a very small sample upon the center of an electron microscope grid. This electrostatic precipitator uses a tritium source to supply the ions which are accelerated by a battery provided electro-potential through a biased aperture into the aerosol channel and toward a grounded electron microscope grid. Since sample flow rates may not exceed 10 cc/min for proper operation and since the total sample is deposited upon only about 0.5 mm^2 of the grid substrate, the instrument is particularly desirable for obtaining small samples of highly radioactive aerosols. The distribution of particles on these samples is related to particle size and must be carefully analyzed.

An automatic electrical particle counter and size analyzer has been developed by Whitby and Clark[169] (Whitby Aerosol Analyzer, Thermo-Systems, Inc , St. Paul, Minn.) for studying aerosols in the diameter range of 0.015 μ to 1 μ. This instrument may also be used as an electrical mobility classifier. Aerosols are charged in a well understood and reproducible manner, and the analysis of the mobility in a concentric precipitator is then relatable to size with the known charging function.

Aerodynamic Samplers

Particularly valuable in relating the size distribution of aerosols to their expected behavior in such situations as inhalation are the sampling devices which

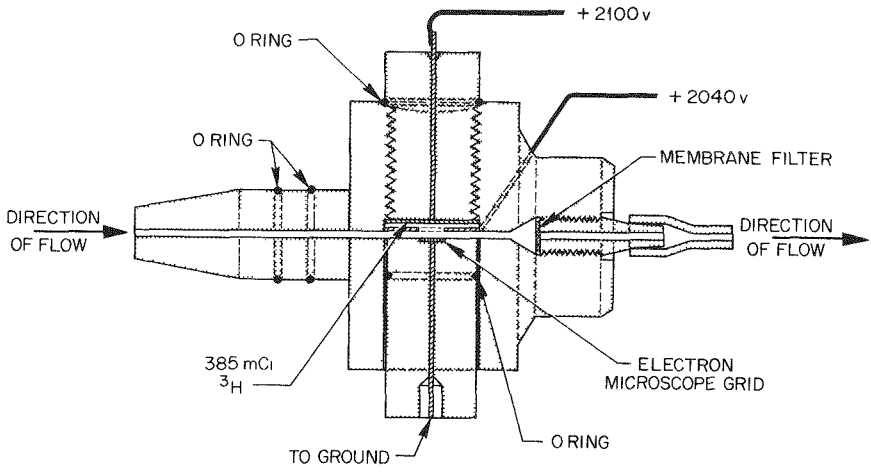

Fig. 18 – An electrostatic precipitator using a tritium source to produce ions for collecting small samples of aerosols for particle size analysis. Aerosol is drawn through the flow channel, a stream of ions produced by the tritium source interacts with the particles under the influence of battery supplied electropotential, and the particles are attracted to the grounded electron microscope grid. (Illustration from Mercer *et al.*[166])

separate size characteristics according to the aerodynamic properties of the particles. These devices usually employ one of the following basic aerodynamic characteristics: (a) sedimentation under gravity or under centrifugal forces, (b) inertial momentum during a change of direction, or (c) natural Brownian motion or diffusion of the particles. The aerodynamic analyses based upon the first two of these characteristics are directly comparable; the third, diffusional characteristics, are different.

One of the most popular of the inertial separators, the cascade impactor, was initially invented by May.[170] Methods of calibration, manner of use, and theory of operation of this instrument have been developed and described by a number of authors including May,[170] Davies *et al.*,[171-172] Ranz and Wong,[173] Mercer,[174-181] and others.[182-197] An aerosol sample is drawn at a constant rate through a series of successively smaller round holes or rectangular slits which have a collection surface very close to the exit and perpendicular to the direction of flow. At each stage the aerosol particles must make a right angle change in direction to follow the air streams; larger particles are unable to negotiate the turn and impact upon the collector. A cross-sectional view of a seven-stage, round-jet cascade impactor is shown in Fig. 19. The average size that will be collected at each stage is successively smaller. An efficient filter usually serves as the final stage to collect all of the smaller particles which successfully pass through the impactor. The graded samples of the aerosol obtained are analyzed with respect to the total amount of material on each collection surface.

Usually a suitable mathematical function is fitted with these impactor data to describe the aerodynamic size distribution. The collection efficiency of each stage depends upon a number of factors: (a) the linear velocities of the air

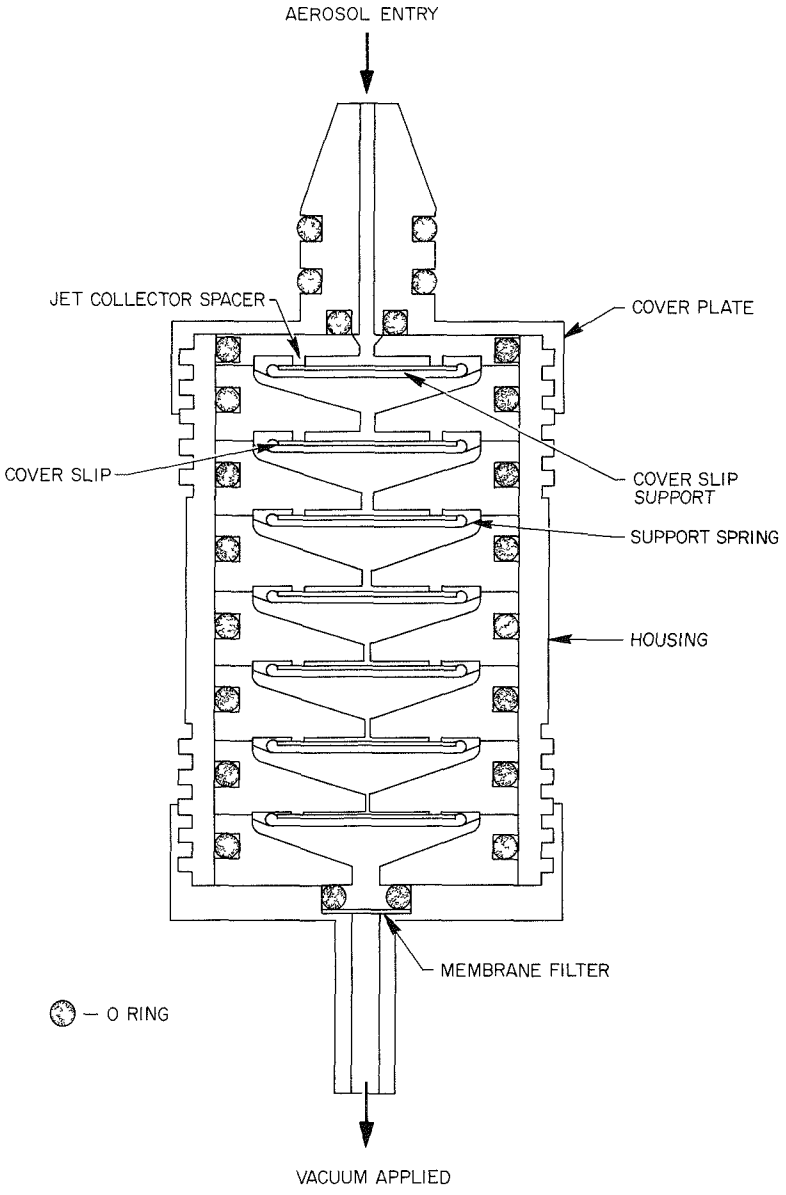

Fig. 19 – A cross-sectional view (not necessarily to scale) of the seven-stage round-jet cascade impactor designed to collect small aerosol samples at flow rates between 50 cc/min and 150 cc/min.

streams (usually described by a hypothetical average, U_0, equal to the volumetric flow rate divided by the area of the opening), (b) the jet opening shape and size (conveniently described by the jet width for slits or the jet diameter for holes, either of which may be called W), (c) the aerosol particle shape, (d) the particle size (diameter, D, if the particle is spherical) (e) the particle density ρ, (f) the slip correction, C (important for smaller particles) (g) the air viscosity, η, (h) the jet-to-collector separation distance, S, and (i) the jet depth, J Ranz and Wong[173] used the dimensionless parameter ψ defined for spherical particles as

$$\psi = \frac{\rho D^2 U_0 C}{18\eta W} \tag{6}$$

and plotted collection efficiency versus $\sqrt{\psi}$ The parameter, $\sqrt{\psi}$, is proportional to the particle diameter Based upon the principles of physical similarity and dimensional analysis, an efficiency curve of this type will be applicable to another impactor stage of the same type but of a different size which has the same ratios, S/W and J/W, and conforms in other ways as to physical similarity The size separation that occurs is not perfect in that the collection efficiency of each stage gradually goes from zero to unity over a small size range One useful technique for analyzing data involves use of the "effective cut-off" particle size, defined as the particle size for which the collection efficiency of a particular impactor stage is equal to 0 5 For simplicity the assumption is made that only particles larger than the effective cut-off size are collected by the impactor stage This method was used by Ranz and Wong,[173] and Mitchell and Pilcher [184] It also has been compared with other methods and recommended by Mercer [179]

The cascade centripeter[198–200] is a newer inertial separator which has been popular in health physics work It is essentially a cascade impactor in which the impaction stages are replaced by small holes in filtered collection chambers so that the undesirable buildup of material that may occur on the collection plates of an impactor is avoided These separators can operate for long periods without disturbing the particle separation characteristics of the stages

Devices which employ sedimentation under the action of gravity for dynamical size separation are known as elutriators and are of two basic types (1) vertical in orientation[201–202] and (2) horizontal in orientation [203–204] The vertical elutriation of particles settling in an upward stream of air has been used in such devices as the Roller Particle Size Analyzer (American Instrument Co , Silver Springs, Maryland) [201] Particles falling at a speed greater than the speed of the upward air streams cannot leave a given elutriation column By using a series of such columns with different diameters or by changing the air flow rate, particles of different sizes can be separated, however, the size separation is not sharp since the velocity profile of upward air approaches a parabolic form Vertical elutriators are not practicable in size classification of sub-micronic aerosols Rectangular horizontal elutriators have been used for aerodynamic size

distribution analyses[204] with the dusty air entering in a thin layer near the top of the channel and clean air entering in laminar flow near the bottom. The particles fall to the bottom of the channel as they flow through and deposit in a spectrum based upon their sedimentation velocities.

Since gravitational settling is inefficient for particles smaller than about 0.5 μ, centrifugal forces are needed in dynamical classification of submicronic aerosols.[205] Instruments using centrifugal separation vary from very simple one-stage cyclones, which create a fast circular motion of air to remove larger particles,[206-208] to very sophisticated aerosol centrifuges.[209] Simple cyclones are useful in estimating the respirable dust fractions of aerosols.[210-211] Among the best of the centrifugal separators for aerodynamic size distribution analyses

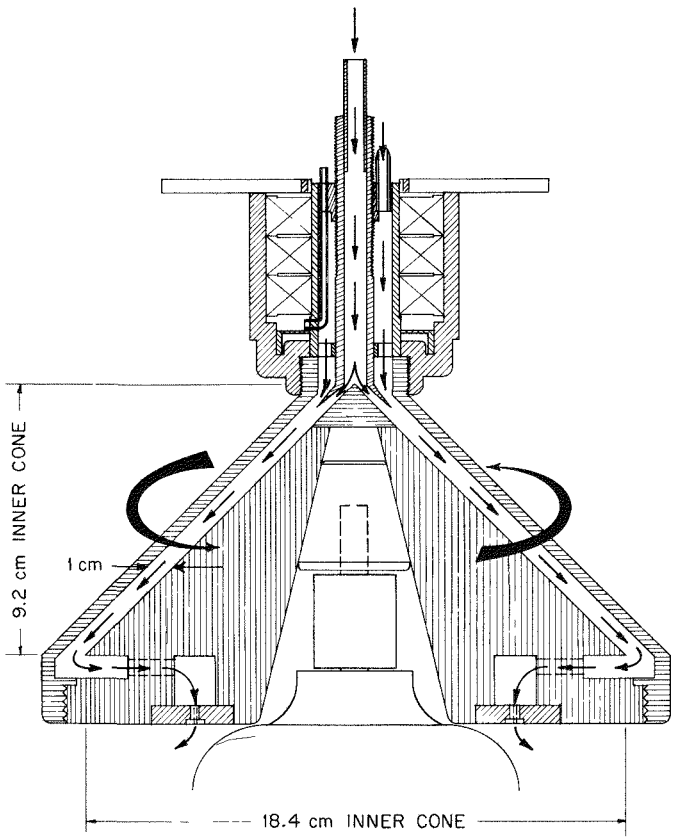

Fig. 20 – Cross-sectional view of the conifuge designed by Tillery showing the air flow patterns. Aerosol samples are drawn into the conifuge at the top through the central stationary tube; clean air is drawn into the concentric tube through an off-centered inlet. The aerosols are separated into a spectrum based upon their dynamical character as they "fall" to the outer wall of the flow channel in the spinning cone. (From Tillery[214-215])

is the conifuge invented by Sawyer and Walton[212] and improved upon and used by Keith and Derrick,[213] Tillery,[214-215] Stober,[216] and others[217] A cross-sectional view of the conifuge designed by Tillery[214-215] is shown in Fig 20 Dusty air enters the open channel in the spinning cone as a thin layer separated from the outer wall by clean air in laminar flow As the aerosol moves down the channel, a centrifugal field produced by the rotor turning at several thousand revolutions per minute, causes the suspended particles to "fall" to the outer wall onto a collection liner or to carefully placed electron-microscope grids Since larger particles fall to the wall sooner than the smaller particles, the deposit consists of a spectrum of particles separated with respect to their aerodynamic properties A poorer type aerosol centrifuge is the Goetz Aerosol Spectrometer[218-221] The name spectrometer is misleading because particles are not actually separated into their respective size groups by this machine Some investigators, however, have used this instrument in experimental studies[222-225] A more advanced centrifugal aerosol spectrometer has recently been designed by Stober[226]

Dynamical measurements of the diffusion properties of aerosols yield a different aerodynamic character because the diffusion coefficients are independent of particle density or mass and depend only upon particle size[227] Instruments and methods for studying diffusion extend from single diffusion tubes as first described by Townsend[228] to elaborate parallel plate diffusion batteries[229-231] Aerosols of very small particles drawn through a diffusion tube or battery are forced to travel a relatively long distance in a confined channel or channels Since the particles are in constant random Brownian motion, many hit the wall of the channel and are firmly held by adhesive and other forces More of the smaller particles are removed from the air stream since they have larger diffusion coefficients, however, separation between sizes is not sharp

Aerosol Particle Size and Interpretations

Particle size analysis has been important in many applied and analytical fields Methods of measuring size distributions, techniques for analyzing the results, and mathematical consideration of the statistical factors have been the subject of many symposia and conferences[232-235] and publications[236-252]

A particle size distribution is commonly treated by dividing a sample of particles into a number of distinct size classes When sizing has been completed, the observed step-like distribution is a rough version of the more smooth distribution of the large population from which the sample was taken For this reason, the observed data are best presented in the form of a histogram showing clearly their piecemeal character The raw data histogram may be standardized with respect to interval size by dividing the number of particles in each interval by the size of that interval The interval sizes are often not equal Greater generalization of the histogram is accomplished by dividing these standardized

values by the total number of particles in the sample to yield a normalized histogram, as shown in Fig. 21. The normalized histogram is a step-like depiction of a particle size distribution function (probability density), C(D):

$$C(D) = \frac{dF(D)}{dD} \tag{7}$$

(which has units of fraction of the distribution per unit interval size) in which D is the particle diameter and F(D) is the cumulative fraction of particles with diameters less than size D. When C(D) or the normalized histogram are integrated with respect to size over all sizes the result is unity. A distribution

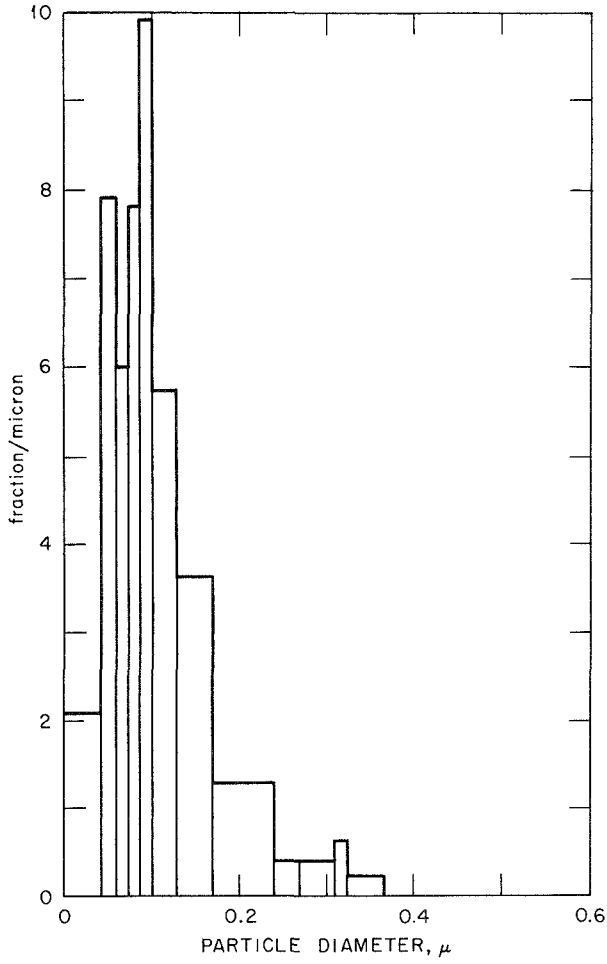

Fig. 21 – An example of a normalized histogram constructed from the data of the sizes of particles in an aerosol sample.

function commonly employed to describe aerosol size distributions is the log-normal distribution given by

$$C(D) = \frac{1}{D\sqrt{(2\pi)}\,\ell n\sigma_g} e^{\frac{(\ell n\, D - \ell n\, CMD)^2}{2(\ell n\sigma_g)^2}} \qquad 0 < D < \infty \tag{8}$$

with ℓn the natural logarithm, D the particle size, CMD the median diameter of the distribution (count median diameter or geometric mean), and σ_g the geometric standard deviation of the distribution Hatch and Choate,[16] demonstrate that any characteristic of the particles in a population which is proportional to the q-th power of the diameter can also be described by a log-normal distribution* with the same geometric standard deviation as the size distribution and with a median diameter, D_q, given by

$$\ell n\, D_q = \ell n\, CMD + q(\ell n\sigma_g)^2 \,. \tag{9}$$

For example, the volume distribution of spherical particles with a number distribution that is log-normal is also log-normal with median D_q given by Eq 9 with q = 3 as in Eq 1 A representative log-normal distribution is shown in Fig. 1 for a distribution with CMD equal to 1.0 μ and geometric standard deviation equal to 2 0. Also included in Fig. 1 are the positions of various other characteristics of the distribution.

Since aerosol particles of unknown shape and properties are studied with many different instruments and techniques, the "sizes" which are reported or studied may not be easily relatable even though they may all be called diameters. Unknown particle shapes will especially lead to various interpretations, since a simple measure of diameter implies sphericity and lacks the suitable shape factors necessary to provide accurate descriptions of the properties of irregular particles [253-254] In optical microscopy, many conventions are employed to decide upon a linear measure for irregular particles. A useful convention has been to measure the diameter of a circular area which is equal to the observed two-dimensional projected area. These projected area diameters can be shown by Cauchy's Theorem to have mean values that are directly relatable to the surface areas of the particles assuming the particles are randomly oriented [255] Clearly, this measure of diameter is not the same as that used if the particles are measured by electrostatic means, by light scattering, or by sedimentation or inertial separation. All of these methods may yield very different values for a single particle and all may be called the particle diameter

*It has been customary in particle size analysis to express all such distributions as a function of particle size This can be the source of some confusion For example, a mass distribution is expressed in units of fraction of the mass per unit particle size interval

To this often confusing situation must be added the different mean or median diameters used to describe distributions of sizes. These include, among others, the volume median diameter (VMD), the diameter of average volume ($\bar{D}_v$) (not to be confused with the mean of the volume distribution), the mass median diameter (MMD), the diameter of average mass ($\bar{D}_m$) (not to be confused with the mean of the mass distribution), the surface area median diameter (AMD), the diameter of average surface area ($\bar{D}_a$) (not to be confused with the mean of the surface area distribution), the count median diameter (CMD), the mean diameter ($\bar{D}$), the modal diameter (D_m), the mass median aerodynamic diameter (MMAD), and the activity median aerodynamic diameter (AMAD) (for radioactive aerosols)

In addition there are at least two different "aerodynamic equivalent diameters" commonly used The first, D_{aer1}, which was described earlier, is the "diameter of a unit density sphere with the same settling velocity as the particle in question" and was recommended by the Task Group on Lung Dynamics [11] This is different from the Stokes Diameter, defined as

$$D_{st} \equiv \sqrt{\left(\frac{18\eta v}{C\rho g}\right)} \tag{10}$$

which is equivalent to the real diameter, D_{real}, of a spherical particle In equation 10 η is the viscosity of the gas (air), ρ the particle density, v the particle settling velocity, C the slip correction, and g the acceleration due to gravity For convenience D_{real} and D_{st} may be treated as mathematically equivalent The relationship between the real diameters of spheres of various densities and the aerodynamic equivalent diameters, D_{aer1}, is shown in Fig 22

The slip correction, C, is a semiempirical factor that corrects the Stokes Law of viscous resistance for the effect of "slip" between the air molecules when the aerosol particles are almost as small or smaller than the free paths of the air molecules [256] The slip correction is approximated for spheres by[257]

$$C = 1 + A\left(\frac{2\lambda}{D_{real}}\right) \tag{11}$$

with

$$A = \alpha + \beta e^{-\gamma\left(\frac{D_{real}}{2\lambda}\right)}$$

with λ the mean free path of the gas molecules, $\alpha \approx 1\ 26$, $\beta \approx 0\ 45$, and $\gamma \approx 1\ 08$ At sea level (760 mm Hg) the mean free path, λ, is equal to about 0 0646 μ for air at 21°C

The second common "aerodynamic diameter", D_{aer2}, is one used by Mercer[26] and other investigators in working with impactors Since it is not

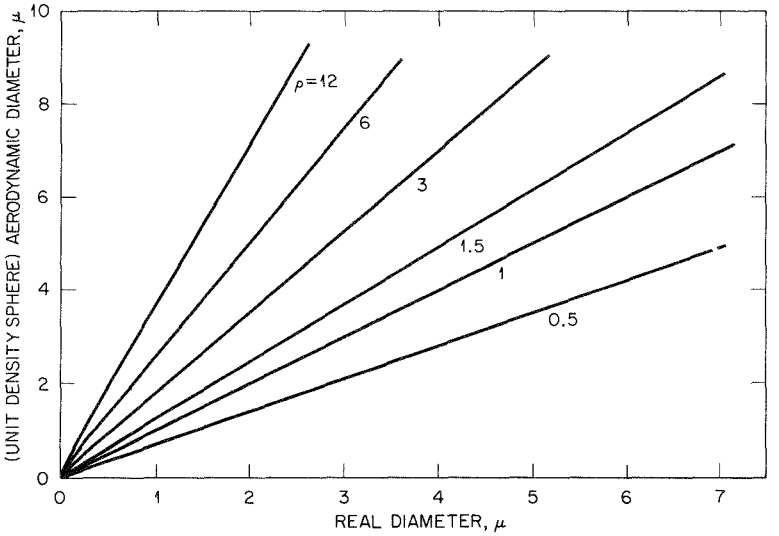

Fig. 22 – The relationship between the real diameters of spherical particles of various densities and the aerodynamic equivalent diameters, D_{aer1}, which are diameters of the equivalent unit density spheres, shown in micron units.

possible to differentiate with a cascade impactor between two spherical particles, one of density ρ_1 and diameter D_1 and the other of density ρ_2 and diameter D_2 if the following equality exists:

$$D_1\sqrt{(\rho_1 C_1)} = D_2\sqrt{(\rho_2 C_2)} \tag{12}$$

(with C_1 the slip correction for the first particle and C_2 the slip correction for the second particle), the aerodynamic equivalent diameter, D_{aer2}, has been defined for spherical particles as

$$D_{aer2} \equiv D_{real}\sqrt{(\rho C)} \tag{13}$$

with D_{real} the real diameter of the spherical particle, ρ its density, and C its slip correction.

Since C depends upon the real diameter, the real diameter of a spherical particle of known aerodynamic diameter D_{aer2} cannot be explicitly stated. However, if it is assumed, for simplicity, that the slip correction is satisfactorily described by Eq. 11 with A = 1.26, then the real diameter is given by (with diameters in micron units):

$$D_{real} = \sqrt{\left[\frac{(D_{aer2})^2}{\rho} + (A\lambda)^2\right]} - A\lambda \tag{14}$$

and D_{aer1} and D_{aer2}, in micron units, are approximately related as:

$$D_{aer1} = \sqrt{[(D_{aer2})^2 + (A\lambda)^2]} - A\lambda \ . \tag{15}$$

If two investigators, one at sea level (*e.g.*, Los Angeles; New York) and the other at 5500 feet above sea level (*e.g.*, Albuquerque; Denver) both measure the aerodynamic equivalent diameters, D_{aer2}, of the same particle, they will obtain different values, since the mean free path of air molecules is about 20% different with a concomitant difference in the slip correction. If, however, these two observers measure different particles and obtain the same value for D_{aer2}, they will be predicting correctly the behavior of these particles for their respective locations, but will obtain a different value for D_{aer1}, the equivalent unit density spheres. The relationship between D_{aer1} and D_{aer2} is shown in Fig. 23.

Neither D_{aer1} nor D_{aer2} is a satisfactory "aerodynamic equivalent diameter" for processes that involve diffusion, such as the inhalation of small, submicron particles. The reason is that they both depend upon the particle density as well as the physical size, but the diffusion coefficient does not. In inhalation studies a better definition for aerodynamic equivalent diameter might be "the equivalent unit density sphere in the process under consideration," whether it be sedimentary, inertial, or diffusional.

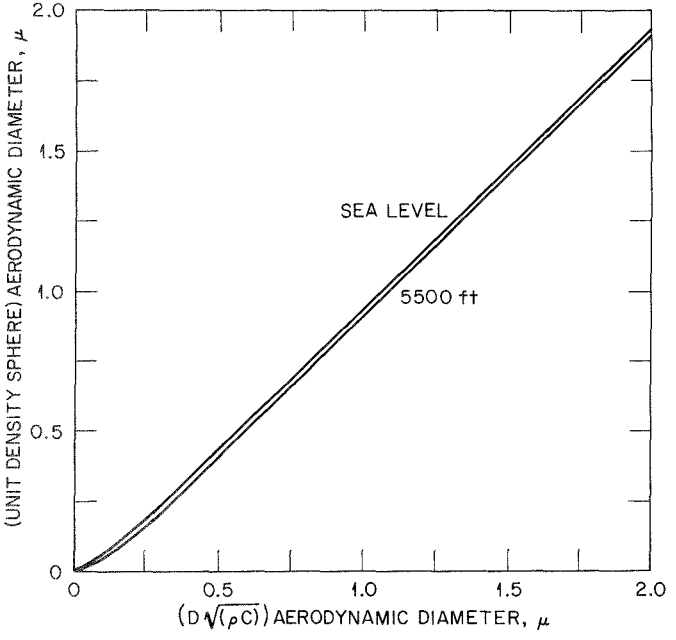

Fig. 23 – The relationship between the two aerodynamic equivalent diameters, D_{aer1}, which is the diameter of the equivalent unit density sphere, and D_{aer2}, which is equal to $D_{real}\sqrt{(\rho C)}$, shown in micron units for two altitudes.

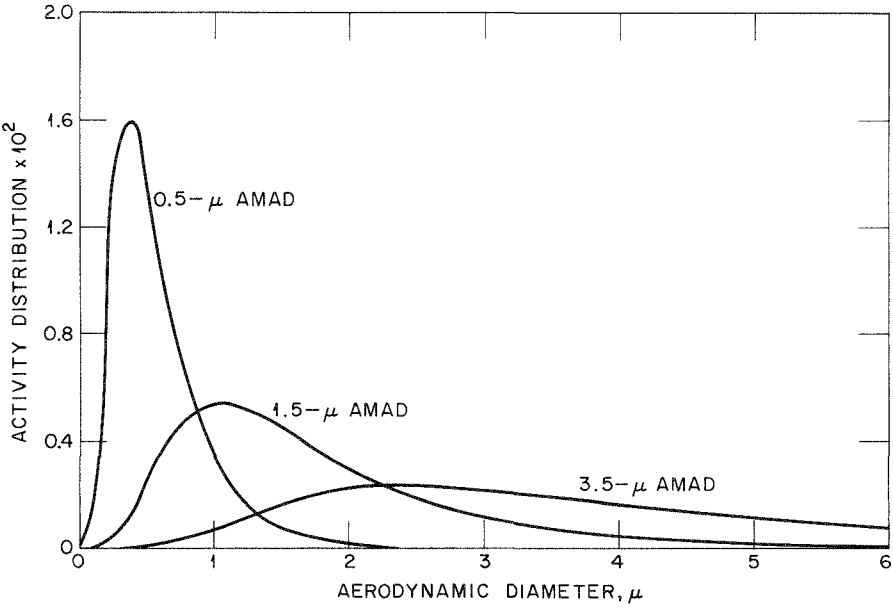

Fig. 24 – Activity distributions of spherical particles of $^{239}PuO_2$ (ρ = 11.5) which are log-normal for aerodynamic sizes (D_{aer2}) plotted for various AMAD with σ_g = 1.8.

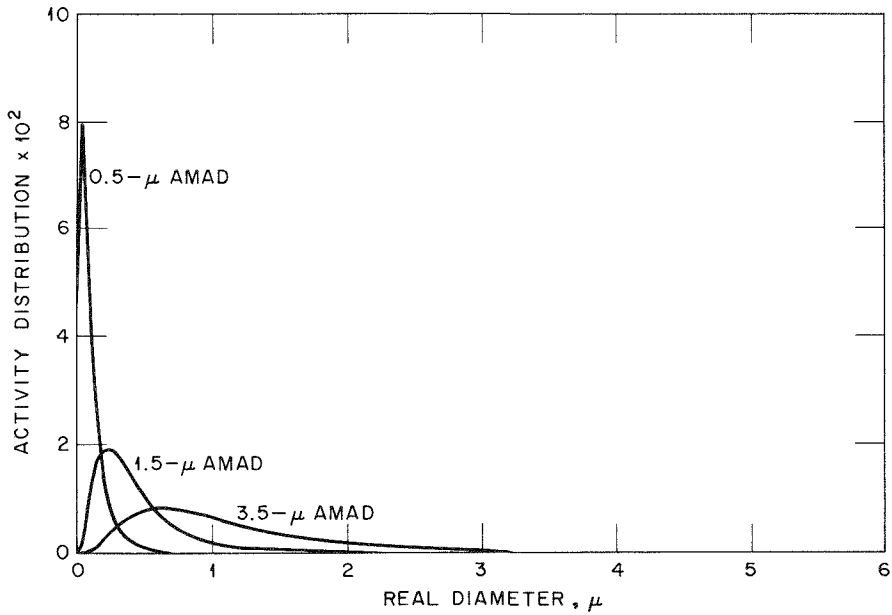

Fig. 25 – Activity distribution of aerosols of spherical particles of $^{239}PuO_2$ (ρ = 11.5) which are log-normal for aerodynamic sizes (D_{aer2}) plotted vs. real sizes for various AMAD with σ_g = 1.8.

If a respirable aerosol is log normally distributed with respect to aerodynamic equivalent diameters, its distribution of real sizes is clearly not log-normal. As a useful approximation, the two distributions may be satisfactorily described as log-normal, but their medians and geometric standard deviations will differ. Shown in Fig. 24 are some log-normal activity distributions of spherical particles of plutonium oxide (ρ = 11.5) plotted with respect to aerodynamic diameters, D_{aer2}. The activity distribution of these same aerosols are plotted with respect to real diameter in Fig. 25.

Clearly, caution is advisable when the term *diameter* is used to describe aerosols. The various "sizes" which are used in the description of aerosols must be properly interpreted in terms of the context, the techniques, and devices used for their determination, the manipulation involved in the applicable calculations, and the conventions involved.

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REFERENCES

1. W. J. Megaw and R. D. Wiffen, The generation of condensation nuclei by ionising radiation. *Geofis. Pura Appl.*, 50 (1961) 118–128.
2. N. A. Renzetti and G. J. Doyle, Photochemical aerosol formation in sulfur dioxide-hydrocarbon systems. *Intern. J. Air Water Pollution*, 2 (1960) 327–345.
3. G. J. Madelaine, Formation et évolution des aerosols dans l'air filtre et dans l'air naturel Action de la radioactivité. Doctoral Thesis, Commissariat a l'Energie Atomique (France) CEA-R-3614, 1968.
4. J. Bricard, F. Billard, and G. Madelaine, Formation and evolution of nuclei of condensation that appear in air initially free of aerosols. *J. Geophys. Res.*, 73 (1968) 4487–4496.
5. M. J. Knott and M. Malanchuk, Analysis of foreign aerosol produced in NO_2-rich atmospheres of animal exposure chambers. *Am. Ind. Hyg. Assoc. J.*, 30 (1969) 147–152.
6. G. Pfefferkorn, Photochemische Bildung Schwerflüchtiger Tröpfchen aus organischen Dämpfen in Luft. *Staub Reinhaltung Luft*, 27 (1967) 138–140.
7. R. A. Mugele and H. D. Evans, Droplet size distribution in sprays. *Ind. Eng. Chem.*, 43 (1951) 1317–1324.
8. V. K. LaMer and R. Gruen, A direct test of Kelvin's equation connecting vapour pressure and radius of curvature. *Trans. Faraday Soc.*, 48 (1952) 410–415.
9. C. Orr, F. K. Hurd and W. J. Corbett, Aerosol size and relative humidity. *J. Colloid Sci.*, 13 (1958) 472–482.
10. M. J. Pilat and R. J. Charlson, Theoretical and optical studies of humidity effects on the size distribution of hygroscopic aerosol. *J. Rech. Atmospheriques*, 2 (1966) 165–170.

11. P. E. Morrow (Chairman, Task Group on Lung Dynamics), Deposition and retention models for internal dosimetry of the human respiratory tract. *Health Phys*, 12 (1966) 173–208
12. C. C Snead and J T. Zung, The effects of insoluble films upon the evaporation kinetic of liquid droplets *J Colloid Interface Sci*, 27 (1968) 25–31.
13 P A Nelson and W. F. Stevens, Size distribution of droplets from centrifugal spray nozzles *A I Ch E. Journal*, 7 (1961) 80–86.
14. H. C. Lewis, D G. Edwards, M. J. Goglia, R I Rice, and L. W Smith, Atomization of liquids in high velocity gas streams *Ind Eng Chem.*, 40 (1948) 67–74
15. T T. Mercer, R. F. Goddard, and R L. Flores, Output characteristics of several commercial nebulizers *Ann. Allergy*, 23 (1965) 314–326.
16 T. Hatch and S. P. Choate, Statistical description of the size properties of non-uniform particulate substances *J Franklin Inst*, 207 (1929) 369–387.
17. T. T. Mercer, Aerosol production and characterization – some considerations for improving correlation of field and laboratory derived data. *Health Phys*, 10 (1964) 873–887.
18. Lord Rayleigh, On the equilibrium of liquid conducting masses charged with electricity. *Phil Mag*, 14 (1882) 184–186.
19 K. T. Whitby and B. Y H. Liu, The electrical behavior of aerosols. In, *Aerosol Science*, pp 59–86, C N Davies (ed). Academic Press Inc , New York, 1966.
20. K. T. Whitby, Generator for producing high concentrations of small ions. *Rev. Sci Instr*, 32 (1961) 351–355.
21 A-L. Soong, The Charge on Latex Particles Aerosolized from Suspensions and Their Neutralization in a Tritium De-Ionizer. M.S. Thesis, University of Rochester, Rochester, New York, 1968.
22. Lord Rayleigh, On the instability of jets. *London Math Soc. Proc.*, 10 (1878–1879) 4–13.
23. R A. Castleman, The mechanism of the atomization of liquids. *Bur Std. J Res*, 6 (1931) 369–376
24. P H. Schweitzer, Mechanism of disintegration of liquid jets *J. Appl. Phys.*, 8 (1937) 513–521.
25 A. C. Merrington and E. G. Richardson, The break-up of liquid jets. *Proc Phys. Soc. (London)*, 59 (1947) 1–13.
26 T T. Mercer, M. I Tillery, and H. Y Chow, Operating characteristics of some compressed air nebulizers *Am. Ind. Hyg Assoc J.*, 29 (1968) 66–78.
27. L. Dautrebande, *Microaerosols*, pp. 7–22, Academic Press Inc., New York, 1962.
28. K. E. Lauterbach, A. D Hayes, and M. A. Coelho, An improved aerosol generator. *A.M A. Arch Ind. Health*, 13 (1956) 156–160.
29. T. T. Mercer, M. I Tillery, and M. A. Flores, Operating Characteristics of the Lauterbach and Dautrebande Aerosol Generators, AEC Research and Development Report LF-6. Lovelace Foundation for Medical Education and Research, Albuquerque, N. Mex., 1963.
30. B. M. Wright, A new nebulizer *Lancet*, 2 (1958) 24–25.
31. G. J. Newton, J. E. Bennick, and S Posner, A miniature high output aerosol generator In, Selected Summary of Studies on the Fission Product Inhalation Program from July 1965 Through June 1966, pp. 29–35 AEC Research and Development Report LF-33, Lovelace Foundation for Medical Education and Research, Albuquerque, New Mexico, 1966
32. K. Sollner, The mechanism of the formation of fogs by ultrasonic waves *Trans Faraday Soc*, 32 (1936) 1532.
33. J. N. Antonevich, Ultrasonic atomization of liquids. In, *Proceedings of the National Electronics Conference*, pp. 798–807. Chicago, Illinois, October 7–9, 1957.

34 S. N. Muromtsev and V. P. Nenashev, The study of aerosols III. An ultrasonic aerosol atomizer. *J. Microbiol Epidemiol. Immunobiol.* (USSR) (English Transl.), 31 (1960) 1840–1846.
35. B. I Il'in and O. K. Eknadiosyants, Nature of the atomization of liquids in an ultrasonic fountain. *Soviet Phys Acoust.* (English Transl.), 12 (1967) 269–275.
36 R. M. G Boucher and J Kreuter, The fundamentals of the ultrasonic atomization of medicated solutions. *Ann Allergy,* 26 (1968) 591–600.
37 G. J Newton, Fission Product Inhalation Laboratories, Lovelace Foundation for Medical Education and Research, unpublished observation, 1968.
38. T. T. Mercer, R. F. Goddard, and R. L. Flores, Output characteristics of three ultrasonic nebulizers *Ann Allergy,* 26 (1968) 18–27.
39. F. R. Gibb and P E Morrow, Alveolar clearance in dogs after inhalation of an iron-59 oxide aerosol. *J Appl Physiol*, 17 (1962) 429–432.
40. M. E. McKnight and R W. E. Norgon, A Study of the Exchange Characteristics of Montmorillonite Clay for Fission Product Cations for Use in the Generation of Insoluble Aerosols, AEC Research and Development Report LF-37. Lovelace Foundation for Medical Education and Research, Albuquerque, N.M., 1967.
41. D. K Craig, An Investigation of the Interactions that Occur Between Radionuclides and Aerosols in the Respirable Size Range, AEC Research and Development Report UR-636, Thesis, University of Rochester, Rochester, N.Y. 1964.
42 S. C. Stern, J. S. Baumstark, A I Schekman, and R. K. Olson, A simple technique for generation of homogeneous millimicron aerosols *J Appl Phys*, 30 (1959) 952–953.
43. G. M. Kanapilly, O. G. Raabe, and G. J Newton. A new method for the generation of aerosols of spherical insoluble particles, *Am. Ind. Hyg Assoc J.,* 30 (1969) 125. (Abstract).
44. Aerosol Physics Dept., Lovelace Foundation, The production of insoluble aerosols of fused clay contaminated with ^{91}Y, ^{137}Cs, and ^{144}Ce. In, Fission Product Inhalation Program Annual Report 1966–1967, pp. 92–100, R. O. McClellan and F. C. Rupprecht (eds) AEC Research and Development Report LF-38, Lovelace Foundation for Medical Education and Research, Albuquerque, N.M., 1967.
45. V. A. Gordieyeff, Studies on dispersion of solids as dust aerosols. *A.M.A Arch. Ind. Health,* 15 (1957) 510–515
46. W. J Bair and B. J McClanahan, Plutonium inhalation studies *Arch. Environ Health,* 2 (1961) 48–55.
47 B. M. Wright, A new dust-feed mechanism. *J Sci Instr.,* 27 (1950) 12–15.
48 R. L. Dimmick, Jet disperser for compacted powders in the one-to-ten micron range. *A.M.A. Arch Ind Health,* 20 (1959) 8–14
49. J C. Couchman, *Metallic Microsphere Generation* EG&G., Inc. Santa Barbara, California, 1966
50 F. G. Karioris and B. R. Fish. An exploding wire aerosol generator. *J. Colloid Sci*, 17 (1962) 155–161.
51. J D. Holmgren, J. O. Gibson, and C. Sheer, Some characteristics of arc vaporized submicron particulates *J. Electrochem. Soc*, III (1964) 362–369.
52. N. A. Fuchs and A. G. Sutugin, Generation and use of monodisperse aerosols In, *Aerosol Science,* pp. 1–30, C. N. Davies (ed.). Academic Press Inc., New York, 1966.
53. B. Y. H. Liu, *Methods of Generation of Monodisperse Aerosols,* COO-1248-10, Dept. of Mechanical Engineering, University of Minnesota, Minneapolis, Minn., 1967.
54. P. C Reist and W. A. Burgess, Atomization of aqueous suspensions of polystyrene latex particles *J Colloid Interface Sci*, 24 (1967), 271–273.
55. E. B. Bradford and J W. Vanderhoff, Electron microscopy of monodisperse latexes. *J Appl Phys*, 26 (1955) 864–871.

56 E B Bradford, J W Vanderhoff, and T Alfrey, Jr , The use of monodisperse latexes in an electron microscope investigation of the mechanism of emulsion polymerization *J Colloid Sci* 11 (1956) 135–149

57 R H Ottewill and R F Woodbridge, Studies on the electrokinetic behavior of monodisperse silver halide sols *J Colloid Sci* 19 (1964) 606 620

58 Werner Stober and Arthur Fink, Controlled growth of monodisperse silica spheres in the micron size range *J Colloid Interface Sci* 26 (1968) 62 69

59 Jacques M Singer, Carel J Van Oss, and John W Vanderhoff, Radioiodination of latex particles *J Reticuloendothelial Soc* 6 (1969) 281–286

60 H Flachsbart and W Stober, Preparation of radioactively labeled monodisperse silica spheres of colloidal size *J Colloid Interface Sci* 30 (1969) 568–573

61 Otto G Raabe, The dilution of monodisperse suspensions for aerosolization *Am Ind Hyg Assoc J*, 29 (1968) 439–443

62 Kenneth T Whitby and Benjamin Y H Liu, Polystyrene aerosols – electrical charge and residue size distribution *Atmos Environ* 2 (1968) 103–116

63 Irwin B Wilson and Victor K LaMer, The retention of aerosol particles in the human respiratory tract as a function of particle radius *J Ind Hyg Toxicol* 30 (1948) 265–280

64 David Sinclair and Victor K LaMer, Light scattering as a measure of particle size in aerosols *Chem. Rev*, 44 (1949) 245–267

65 Victor K LaMer, Edward C Y Inn and Irwin B Wilson, The methods of forming, detecting, and measuring the size and concentration of liquid aerosols in the size range of 0 01 to 0 25 microns diameter *J Colloid Sci* 5 (1950) 471–497

66 E Rapaport, and S E Weinstock, A generator for homogeneous aerosols *Experientia* 11 (1955) 363–364

67 Lars Lassen, A simple generator for the production of monodisperse aerosols in the size range of 0 15 to 0 70 microns (particle radius) *Z Angew Phys* 4 (1960) 157–159

68 P Mouillat, Production et observation d'aerosols monodisperses application a l'etude de quelques proprietes physiques d'etats disperses *Ann Occupational Hyg* 4 (1962) 275–294

69 E Matijevic, S Kitani, and M Kerker, Aerosol studies by light scattering II Preparation of octanoic acid aerosols of narrow and reproducible size distributions *J Colloid Sci* 19 (1964) 223–237

70 D L Swift, A study of the size and monodispersity of aerosols produced in a Sinclair-LaMer generator *Ann Occupational Hyg*, 10 (1967) 337–348

71 Benjamin Liu, Kenneth T Whitby, and Henry H S Yu, A condensation aerosol generator for producing monodispersed aerosols in the size range, 0 036 μ to 1 3 μ *J Rech Atmospheriques* (1966) 397–406

72 J Edwards and B J Brinkworth, A device for the control of particle size in the LaMer aerosol generator *J Sci Instrum. Ser 2,* 1 (1968) 636 637

73 E Matijevic, W F Espenscheid, and M Kerker, Aerosols consisting of spherical particles of sodium chloride *J Colloid Sci* 18 (1963) 91–93

74 Susumu Kitani and Sohei Ouchi, Preparation of monodisperse aerosols of sodium chloride *J Colloid Interface Sci* 23 (1967) 200–202

75 W R Wolf, Study of the vibrating reed in the production of small droplets and solid particles of uniform size *Rev Sci Instrum* 32 (1961) 1124 1129

76 Bedrich Binek and Blanka Dohnalova, Ein Generator zur Herstellung monodisperser Aerosole aus der flussigen Phase *Staub Reinhaltung Luft* 27 (1967) 492–494

77 Vadim G Drozin, The electrical dispersion of liquids as aerosols *J Colloid Sci* 10 (1955) 158–164

78 B Vonnegut and R Neubauer, Production of monodisperse liquid particles by electrical atomization *J Colloid Sci* 7 (1952) 616–621

79 Edward P Yurkstas and Charles J Meisenzahl, Solid Homogeneous Aerosol Production by Electrical Atomization, AEC Research and Development Report UR 652 University of Rochester Atomic Energy Project, Rochester, New York (1964)

80 W H Walton and W C Prewett, The production of sprays and mists of uniform drop size by means of spinning disc type sprayers *Proc Phys Soc (London)*, 62 (1949) 341 350

81 K R May, An improved spinning top homogeneous spray apparatus *J Appl Phys* 20 (1949) 932–938

82 K R May, Spinning top homogeneous aerosol generator with shock proof mounting *J Sci Instrum.* 43 (1966) 841 842

83 L C Schwendiman, A K Postma, and L F Coleman, A spinning disc aerosol generator *Health Phys* 10 (1964) 947–953

84 R E Albert, H G Petrow, A S Salam, and J R Spiegelman, Fabrication of monodisperse lucite and iron oxide particles with a spinning disk generator *Health Phys* 10 (1964) 933–940

85 G A Sehmel, The density of uranine particles produced by a spinning disc aerosol generator *Am Ind Hyg Assoc J* 28 (1967) 491–492

86 John McK Ellison, Adaptation of the spinning top generator to provide aerosols in the respirable range *Ann Occup Hyg* 10 (1967) 363–367

87 Morton Lippmann and Roy E Albert A compact electric motor driven spinning disc aerosol generator *Am Ind. Hyg Assoc. J*, 28 (1967) 501–506

88 Jennings E Partridge and Harry J Ettinger, Calibration of a Spinning Disc Aerosol Generator and Two Stage Air Samplers AEC Research and Development Report LA-4066 Los Alamos Scientific Laboratory, Los Alamos, New Mexico (1969)

89 A Kajland, M Edfors, L Friberg, and B Holma, Radioactive monodisperse test aerosols and lung clearance studies *Health Phys* 10 (1964) 941–945

90 D V Booker, A C Chamberlain, J Rundo, D C F Muir, and M L Thomson, Elimination of 5 μ particles from the human lung *Nature* 215 (1967) 30–33

91 D C F Muir and C N Davies, The deposition of 0 5 μ diameter aerosols in the lungs of man *Ann Occupational Hyg* 10 (1967) 161–174

92 Roy E Albert, Morton Lippmann, Jack Spiegelman, Anthony Liuzzi, and Norton Nelson, The deposition and clearance of radioactive particles in the human lung *Arch Environ Health* 14 (1967) 10–15

93 Bo Holma, Lung Clearance of Mono and Di Disperse Aerosols Determined by Profile Scanning and Whole-Body Counting A Study on Normal and SO_2 Exposed Rabbits From the Department of Hygiene, Karolinska Institute, and the Department of Environmental Hygiene, National Institute of Public Health, Stockholm, Sweden, 1967

94 Bo Holma, The initial lung clearance of di-disperse (2μ and 7μ) polystyrene particles in rabbits *Arch Environ Health* 17 (1968) 871–873

95 A Black and M Walsh, Production of Uniform Radioactive Particles for Aerosol Inhalation Experiments Part I Particles in the Size Range 1–10 Microns, United Kingdom Atomic Energy Authority Research Group Report, AERE-R 5716 Health Physics and Medical Division, Atomic Energy Research Establishment, Harwell, Berkshire, 1968

96 M J Fulwyler, Electronic separation of biological cells by volume *Science,* 150 (1965) 910–911

97. M. J. Fulwyler, R. B. Glascock, and R. B. Hiebert, Device which separates minute particles according to electronically sensed volume. *Rev. Sci. Instrum.*, 40 (1969) 42–48.
98. M. J. Fulwyler and O. G. Raabe, The ultrasonic generation of monodisperse aerosols. To be presented at the American Industrial Hygiene Conference, Detroit, 1970.
99. L. Strom, The generation of monodisperse aerosols by means of a disintegrated jet of liquid. *Rev. Sci. Instrum.*, 40 (1969) 778–782.
100. D. G. Skinner and S. Boas-Traube, The light-extinction method of particle size estimation. In, *Symposium on Particle Size Analysis, Trans. Inst. Chem. Engrs. (London)*, Suppl., 25 (1947) 57–63.
101. Robert L. Dimmick, Melvin T. Hatch and James Ng, A particle sizing method for aerosols and fine powders. *A.M.A. Arch. Ind. Health,* 18 (1958) 23–29.
102. Jörn Olaf and Klaus Robock, Zur Theorie der Lichtstreuung an Kohle-und Bergepartikeln. *Staub,* 21 (1961) 495–499.
103. B. V. Derjaguin and G. Ja Vlasenko, Flow-ultramicroscopic method of determining the number concentration and particle size analysis of aerosols and hydrosols. *J. Colloid Sci.,* 17 (1962) 605–627.
104. J. Raymond Hodkinson, Light scattering and extinction by irregular particles larger than the wavelength. In, *Proceedings of Interdisciplinary Conference on Electromagnetic Scattering,* pp. 87–100. Pergamon Press, New York, N.Y., 1963.
105. J. Raymond Hodkinson, Die Interpretation von Berechnungen nach der Mie-Theorie für absorbierende Kugeln im Hinblick auf die Staubmessung nach der Streulichtmethode. *Staub,* 23 (1963) 374–378.
106. J. R. Hodkinson, The physical basis of dust measurement by light scattering. In, *Aerosols,* pp. 181–194. Gordon and Breach Science Publishers, Inc., New York, 1965.
107. M. Kerker, E. Matijevic, W. F. Espenscheid, W. A. Farone, and S. Kitani, Aerosol studies by light scattering. I. Particle size distribution by polarization ratio method. *J. Colloid Sci.,* 19 (1964) 213–222.
108. Frank T. Gucker, Jr., Determination of concentration and size of particulate matter by light scattering and sonic techniques. *Proc. Natl. Air Pollution Symposium,* 1 (1949) 14–26.
109. J. Raymond Hodkinson and Judith R. Greenfield, Response Calculations for light-scattering aerosol counters and photometers. *Appl. Opt.,* 4 (1965) 1463–1474.
110. J. Raymond Hodkinson, Zur Theorie des Tyndalloskops. *Staub Reinhaltung Luft,* 26 (1966) 238–239.
111. F. T. Gucker and D. G. Rose, A photoelectronic instrument for counting and sizing aerosol particles. *Brit. J. Appl. Phys.*, Suppl. 3 (1954) 138–143.
112. L. Coutarel, E. Matijevic, M. Kerker, and Chao-Ming Huang, Aerosol studies by light scattering. VI. Preparation and growth of sulfuric acid aerosols. *J. Colloid Interface Sci.,* 24 (1967) 338–343.
113. Kanji Takahashi, Determination of number concentration of poly-dispersed small aerosol particles by turbidity measurement. *J. Colloid Interface Sci.,* 24 (1967) 159–163.
114. Alfred C. Holland and James Stark Draper, Analytical and experimental investigation of light scattering from polydispersions of Mie particles. *Appl. Opt.,* 6 (1967) 511–518.
115. L. G. Wilson and P. Cavanagh, A stereo-ultramicroscope for studying sub-micron aerosols. *Atmos. Environ.,* 4 (1969) 47–53.
116. Kanji Takahashi and Shigehisa Iwai, Estimation of size distribution of small aerosol particles by light-scattering measurement. *J. Colloid Interface Sci.,* 23 (1967) 113–119.

117 Pui Kum Lee and Victor K LaMer, A forward angle light scattering camera for determining size distribution in aerosols *Rev Sci Instrum.* 25 (1954) 1004–1010
118 Samuel H Maron and Max E Elder, Determination of latex particle size by light scattering II Higher-order tyndall spectra *J Colloid Sci* 18 (1963) 199–207
119 T D Proctor, A laser technique for the measurement of aerosols *J Sci Instrum Ser 2* 1 (1968) 631–635
120 J R Hodkinson, The optical measurements of aerosols In, *Aerosol Science* pp 287–357, C N Davies (ed) Academic Press, Inc , New York, 1966
121 H C Van DeHulst, *Light Scattering by Small Particles* John Wiley and Sons, Inc , London, 1957
122 W H M Robins, A microscope method of measuring thickness of fine particles In, *The Physics of Particle Size Analysis Brit J Appl Phys* , Suppl No 3, pp 189–191, 1954
123 G L Fairs, The use of the microscope in particle size analysis *Chem Ind (London)*, 62 (1943) 374–378
124 W H Walton, The application of electron microscopy to particle size measurement *Trans Inst Chem. Engrs (London)*, Suppl 25 (1947) 64–76
125 W N Maclay and E M Gindler, Latex particle size determinations by electron microscope techniques *J Colloid Sci* 18 (1963) 343–352
126 R M Fisher, J S Lally, and Marie H Greider, Electron microscopy. *Anal Chem* 40 (1968) 554R-560R
127 A D Joffe, Comparative surface areas of dust samples as measured by light and electron microscopes *Brit J Appl Phys* 14 (1963) 429–431
128 James W May, Viscous impingement filters In, *Air Pollution, Proceedings of the United States Technical Conference on Air Pollution* McGraw Hill Book Company, Inc , New York, N Y , 1952
129 Leslie A Chambers, Filter media for air sampling *Am Ind Hyg Assoc Quart*, 15 (1954) 290–296
130 C Y Chen, Filtration of aerosols by fibrous media *Chem. Rev* 55 (1955) 595–623
131 S C Stern, H W Zeller, and A I Schekman, The aerosol efficiency and pressure drop of a fibrous filter at reduced pressure *J Colloid Sci* 15 (1960) 546–562
132 Josef Pich, Die Filtrationstheorie hochdisperser Aerosole *Staub,* 25 (1965) 186–192
133 K Spurny and J Pich, Analytical methods for determination of aerosols by means of membrane ultrafilters VII Diffusion and impaction precipitation of aerosol particles by membrane ultra-filters *Collection Czech Chem. Commun* , 30 (1965) 2276–2287
134 J Pich, Theory of filtration of highly dispersed aerosols *Collection Czech Chem. Commun* 31 (1966) 3721–3736
135 J Pich, Pressure drop of fibrous filters at small Knudsen numbers, *Ann. Occupational Hyg* 9 (1966) 23–27
136 I B Stechkina and N A Fuchs, Studies on fibrous aerosol filters I Calculation of diffusional deposition of aerosols in fibrous filters *Ann Occupational Hyg* , 9 (1966) 59–64
137 A A Kirsch and N A Fuchs, Studies on fibrous aerosol filters III Diffusional deposition of aerosols in fibrous filters *Ann Occupational Hyg* , 11 (1968) 299–304
138 J Pich, Theory of aerosol filtration by fibrous and membrane filters In, *Aerosol Science* pp 223–285, C N Davies (ed) Academic Press, Inc , New York, 1966
139 K Spurny, Membranfilter in der Aerosologie II Filtrationsmechanismen bei Membranfiltern *Zentr Biol Aerosol Forsch* , 12 (1965/66) 3–56
140 R M Werner and L A Clarenburg, Aerosol filters, Pressure drop across single-component glass fiber filters *Ind Eng Chem. Fundamentals* 4 (1965) 288–293
141 Alexander Goetz, Application of molecular filter membranes to the analysis of aerosols *J Public Health,* 43 (1953) 150–159

142. H. J. Paulus, N. A. Talvitie, D. A. Fraser, and R. G. Keenan, Use of membrane filters in air sampling. *Am Ind. Hyg Assoc. Quart.*, 18 (1957) 267–273.
143 V. Hampl and K. Spurny, Analytical methods for determination of aerosols by means of membrane ultrafilters. VIII. Determination of the mean pore size by gas flow rate measurement. *Collection Czech. Chem. Commun.*, 30 (1966) 1152–1161.
144. Kvetoslav Spurny, Zur Frage der Filtrationsmechanismen bei Membranfiltern. *Staub*, 24 (1964) 250–256.
145. Philip Rosenblatt and Victor K. LaMer, Motion of a particle in a temperature gradient, thermal repulsion as a radiometer phenomenon. *Phys Rev*, 70 (1946) 385–395
146. I. G. Poppoff, Dust sampling by thermal precipitation. *Am Ind. Hyg Assoc Quart*, 15 (1954) 145–151.
147. K. E. Lauterbach, R. H. Wilson, S. Laskin, and D W. Meier, Design of an Oscillating Thermal Precipitator, AEC Research and Development Report UR-199 University of Rochester, Rochester, New York, 1952.
148 Clyde Orr and Roy A. Martin, Thermal precipitator for continuous aerosol sampling *Rev. Sci. Instrum.*, 29 (1958) 129–130.
149. H. H. Watson, The sampling efficiency of the thermal precipitator *Brit J Appl Phys*, 9 (1958) 78–79.
150. J. H. Daniel and F. S. Brackett, An electrical method for investigating the nature and behavior of small, airborne, charged particles *J App. Phys.*, 22 (1951) 542–554.
151. J. H. Daniel and F. S. Brackett, Investigation of small, airborne, charged particles by an electrical method. *A M.A. Arch. Ind. Hyg. Occupational Med.*, 3 (1951) 505 519.
152. T. Gillespie and G. O. Langstroth, An instrument for determining the electrical charge distribution in aerosols. *Can. J Chem.*, 30 (1952) 1056–1068.
153. Herbert H. Yoshikawa, George A. Swartz, John T. MacWaters, and Wade L. Fite, Electrostatic particle size analyzer. *Rev. Sci Instrum.*, 27 (1956) 359–362.
154. G. Langer, Electrostatic Classification of Submicron Airborne Particles, AEC Progress Report, ARF 3187-8 Armour Research Foundation of Illinois Institute of Technology, Germantown, Md., 1963.
155. Joseph I. Masters, An aerosol analyzer. *Rev. Sci. Instrum.*, 24 (1953) 586–588
156. B. L. Hinkle, Clyde Orr, Jr., and J. M. DallaValle, A new method for the measurement of aerosol electrification. *J Colloid Sci.*, 9 (1954) 70–80.
157. Thomas T. Mercer, Charging and Precipitation Characteristics of Sub-micron Particles in the Rohmann Electrostatic Particle Separator, AEC Research and Development Report UR-475. University of Rochester, Rochester, New York, 1957.
158. G. Langer and J. L. Radnik, Development and preliminary testing of a device for electrostatic classification of submicron airborne particles. *J. Appl. Phys*, 22 (1961) 955–957.
159. Kenneth T. Whitby and Carl M. Peterson, Electrical neutralization and particle size measurement of dye aerosols. *Ind. Eng. Chem. Fundamentals*, 4 (1965) 66–72.
160. Jess W Thomas and David Rimberg, Eine einfache Methode zur Messung der mittleren elektrischen Ladung eines monodispersen Aerosols *Staub Reinhaltung Luft*, 27 (1967) 354–357.
161. Harry J. White, *Industrial Electrostatic Precipitation*. Addison-Wesley Publishing Co., Inc., Reading, Mass , 1963
162. H. J. White, Particle charging in electrostatic precipitation. *Trans Am Inst Elec Engrs.*, 70 (1951) 1–6.
163. E. C Barnes, Atmospheric sampling by electrostatic precipitation In, *Air Pollution, Proceedings of the United States Technical Conference on Air Pollution*. McGraw-Hill Book Company, Inc., New York, 1953.

164. Kenneth E. Lauterbach, Thomas T. Mercer, Alastair D. Hayes, and Paul E. Morrow, Efficiency studies of the electrostatic precipitator. *A.M.A. Arch. Ind. Hyg. Occupational Med.*, 9 (1954) 69–75.
165. Morton Lippmann, Hugo J. DiGiovanni, Samuel Cravitt, and Pedro Lilienfeld, Lightweight, high-volume electrostatic precipitator survey sampler. *Am. Ind. Hyg. Assoc. J.*, 26 (1965) 485–489.
166. Paul E. Morrow and Thomas T. Mercer, A point-to-plane electrostatic precipitator for particle size sampling. *Am. Ind. Hyg. Assoc. J.*, 25 (1964) 8–14.
167. T. T. Mercer, M. I. Tillery, and M. A. Flores, An Electrostatic Precipitator for the Collection of Aerosol Samples for Particle Size Analysis, AEC Research and Development Report, LF-7. Lovelace Foundation for Medical Education and Research, Albuquerque, N.M., 1963.
168. Benjamin Y. H. Liu, Kenneth T. Whitby and Henry H. S. Yu, Electrostatic aerosol sampler for light and electron microscopy. *Rev. Sci. Instrum.*, 38 (1967) 100–102.
169. Kenneth T. Whitby and William E. Clark, Electric aerosol particle counting and size distribution measuring system for the 0.015 to 1μ size range. *Tellus*, 17 (1966) 573–586.
170. K. R. May, The cascade Impactor: An instrument for sampling coarse aerosols. *J. Sci. Instrum.*, 22 (1945) 187–195.
171. C. N. Davies and Mary Aylward, The trajectories of heavy, solid particles in a two-dimensional jet of ideal fluid impinging normally upon a plate. *Proc. Phys. Soc. (London)*, 64 B (1951) 889–911.
172. C. N. Davies, Mary Aylward, and Dorothy Leacey, Impingement of dust from air jets. *A.M.A. Arch. Ind. Hyg. and Occupational Med.*, 4 (1951) 354–397.
173. W. E. Ranz and J. B. Wong, Jet impactors for determining the particle-size distributions of aerosols. *A.M.A. Arch. Ind. Health Occupational Med.*, 4 (1952) 464–477.
174. T. T. Mercer, On the Calibration of Cascade Impactors, AEC Research and Development Report, LF-1. Lovelace Foundation for Medical Education and Research, Albuquerque, N.M., 1962.
175. T. T. Mercer, M. I. Tillery, and C. W. Ballew, A Cascade Impactor Operating at Low Volumetric Flow Rates, AEC Research and Development Report, LF-5. Lovelace Foundation for Medical Education and Research, Albuquerque, N.M., 1962.
176. T. T. Mercer, The Stage Constants of Cascade Impactors, AEC Research and Development Report, LF-12. Lovelace Foundation for Medical Education and Research, Albuquerque, N.M., 1963.
177. T. T. Mercer, On the calibration of cascade impactors. *Ann. Occupational Hyg.*, 6 (1963) 1–14.
178. T. T. Mercer, The stage constants of cascade impactors. *Ann. Occupational Hyg.*, 7 (1964) 115–125.
179. T. T. Mercer, The interpretation of cascade impactor data. *Amer. Indus. Hyg. Assoc. J.*, 26 (1965) 236–241.
180. T. T. Mercer and H. Y. Chow, Impaction from rectangular jets. *J. Colloid Interface Sci.*, 27 (1968) 75–83.
181. T. T. Mercer and R. G. Stafford, Impaction from round jets. *Ann. Occupational Hyg.*, 12 (1969) 41–48.
182. L. S. Sonkin, A modified cascade impactor. *J. Ind. Hyg.*, 28 (1946) 269–272.
183. Sidney Laskin, Modified Cascade Impactor, AEC Research and Development Report MDDC-1500. University of Rochester, Rochester, New York, 1946.
184. R. I. Mitchell and J. M. Pilcher, Design and calibration of an improved cascade impactor for size analysis of aerosols. In, *Proc. Fifth AEC Air Cleaning Conference*, TID-7551, pp. 67–84 DTS. Dept. of Commerce, Washington, D.C., 1950.

185 Sidney Laskin, The modified cascade impactor In, *Air Pollution Proceedings of the United States Technical Conference on Air Pollution* pp 656 671 McGraw Hill Book Co , Inc New York, 1952
186 J M Pilcher, R I Mitchell, and R E Thomas, The cascade impactor for particle-size analysis of aerosols In, *Proc 42nd Ann Meeting Chem. Spec Mfgrs Assoc Inc* , New York, 1955
187 Ariel A Andersen, New sampler for the collection, sizing and enumeration of viable airborne particles *J Bacteriol* 76 (1958) 471 484
188 R I Mitchell and J M Pilcher, Improved cascade impactor for measuring aerosol particle sizes in air pollutants, commercial aerosols, cigarette smoke *Ind Eng Chem.* 51 (1959) 1039–1042
189 Axel Berner and Othmar Preining, Uber eine neue Eichung des Casella-Kaskadenimpaktors *Staub,* 24 (1964) 292–295
190 A A Andersen, A sampler for respiratory health hazard assessment *Am Ind Hyg Assoc J* , 27 (1966) 160–165
191 B J Wells, an evaluation of the May type of cascade impactor *Health Phys* , 13 (1967) 1001–1013
192 Jerry J Cohen and Donald N Montan, Theoretical considerations, design, and evaluation of a cascade impactor *Am Ind Hyg Assoc J* 28 (1967) 95–104
193 Gottfried Hanel, Bemerkungen zur Theorie der Dusen-impaktoren *Atmos Environ* 3 (1969) 69–83
194 Aerosol Physics Dept , Lovelace Foundation, Analysis of cascade impactor data by a weighted least squares method In, Fission Product Inhalation Program Annual Report 1967–1968, AEC Research and Development Report LF-39, pp 165–171, R O McClellan and F C Rupprecht (eds) Lovelace Foundation, Albuquerque, N M , 1968
195 G W C Tait and J Beal, The integrating impactor A monitor for airborne alpha emitters *Health Phys* , 10 (1964) 279–282
196 J C Couchman, Study of the Use of Cascade Impactors for Analyzing Airborne Particles of High Specific Gravity, Defense Atomic Support Agency Research and Development Report, FZK-236 Defense Atomic Support Agency, Sandia Base, Albuquerque, N M , 1965
197 Dale A Lundgren, A aerosol sampler for determination of particle concentration as a function of size and time *J Air Pollution Control Assoc* , 17 (1967) 225–229
198 R F Hounam, The Cascade Centripeter A Device for Determining the Concentration and Size Distribution of Aerosols, United Kingdom Atomic Energy Authority Research Group Memorandum, AERE-M 1328 Health Physics and Medical Division, Atomic Energy Research Establishment, Harwell, Berkshire, 1964
199 R F Hounam and R J Sherwood, The cascade centripeter A device for determining the concentration and size distribution of aerosols *Am Ind Hyg Assoc J* , 26 (1965) 122–131
200 E Dubois, P Magot-Cuvru, J Y Deysson, M Lutz, H Metivier, and R Bessieres, Etude experimentale D'un selecteur de tailles pour aerosols In, *Assessment of Airborne Radioactivity,* pp 379–392 International Atomic Energy Agency, Vienna, 1967
201 A E Reif, C S White, and M E Giblin, Aerosol production and size analysis with the Roller separator *A M A Arch Indus Health,* 14 (1956) 442–449
202 W H Walton, Theory of size classification of airborne dust clouds by elutriation *The Physics of Particle Size Analysis, Brit J Appl Phys* Suppl 3 (1954) 529–537
203 R J Hamilton and W H Walton, The selective sampling of respirable dust In, *Inhaled Particles and Vapours, I,* pp 465–475, C N Davies (ed) Pergamon Press, New York, N Y , 1961

204 Werner Stober, Zur Bestimmung von Teilchengrossenverteilungen mit einem Horizontal-Elutriator *Staub,* 25 (1964) 221–223
205 A E Jacobsen and W F Sullivan, Centrifugal sedimentation method for particle size distribution *Ind Eng Chem Anal Ed*, 18 (1946) 360–364
206 M Lippmann and W B Harris, Size-selective samplers for estimating "respirable" dust concentrations *Health Phys*, 8 (1962) 155–163
207 Ludwig Leineweber, Auslegung von Zyklonabscheidern nach vorgegebenen Werten fur Grenzkorn, Druckverlust und Durchsatz *Staub Reinhaltung Luft,* 27 (1967) 123–129
208 Hans Breuer, Investigations on the suitability of cyclones for the selective sampling of respirable fine dusts In, *Inhaled Particles and Vapours* II, pp 523–530, C N Davies (ed) Pergamon Press, New York, N Y 1967
209 W Stober and U Zessack, Zur Messung Von Aerosol-Teilchengrossenspektren mit Hilfe von Zentrifugalabscheidern *Zentr Aerosol Forsch*, 13 (1966) 263–281
210 W B Harris and Merril Eisenbud, Dust sampler which simulates upper and lower lung deposition *A M A Arch Ind Hyg Occupational Med*, 8 (1953) 446–452
211 Paul E Morrow, Evaluation of inhalation hazards based upon the respirable dust concept and the philosophy and application of selective sampling *Am Ind Hyg Assoc J*, 25 (1964) 213–236
212 K F Sawyer and W H Walton, The "conifuge" – A size-separating sampling device for airborne particles *J Sci Instrum.*, 27 (1950) 272–275
213 C H Keith and J C Derrick, Measurement of the particle size distribution and concentration of cigarette smoke by the "conifuge" *J Colloid Sci*, 15 (1960) 340–356
214 M I Tillery, Design and Calibration of a Modified Conifuge, AEC Research and Development Report, LF-34 Lovelace Foundation for Medical Education and Research, Albuquerque, N M, 1967
215 M I Tillery, Design and calibration of a modified conifuge In, *Assessment of Airborne Radioactivity,* pp 405–415 International Atomic Energy Agency, Vienna, 1967
216 W Stober, Design and performance of a size-separating aerosol centrifuge facilitating particle size spectrometry in the submicron range In, *Assessment of Airborne Radioactivity,* pp 393–404 International Atomic Energy Agency, Vienna, 1967
217 Helger Hauck and Johann A Schedling, Uber ein modifiziertes Modell einer Konifuge *Staub Reinhaltung Luft,* 28 (1968) 18–29
218 A Goetz and O Preining, The Aerosol Spectrometer and its application to nuclear condensation studies, *Monograph No 5, Physics of Precipitation,* pp 164–183. American Geophysical Union, 1960
219 Werner Stober and Uwe Zessack, Zur Theorie einer Konischen Aerosolzentrifuge *Staub,* 24 (1964) 295–305
220 O G Raabe, Calibration and use of the Goetz aerosol spectrometer In, *Assessment of Airborne Radioactivity,* pp 417–431 International Atomic Energy Agency, Vienna, 1967
221 Emil Baust, Die Verwendbarkeit des Goetzschen Aerosolspektrometers zur Messung von Grossenspektren polydisperser Aerosole *Staub Reinhaltung Luft,* 27 (1967) 180–185
222 F L Ludwig and Elmer Robinson, Size distribution of sulfur-containing compounds in urban aerosols *J Colloid Sci*, 20 (1965) 571–584
223 D K Craig, An investigation of the interactions that occur between I_2 radionuclides and an iron oxide aerosol in the respirable size range *Health Phys*, 12 (1966) 1047–1067
224 F L Ludwig and Elmer Robinson, Variations in the size distributions of sulfur-containing compounds in urban aerosols *Atmos Environ*, 2 (1968) 13–23.

225 Otto G Raabe, The adsorption of radon daughters to some polydisperse submicron polystyrene aerosols *Health Phys* 14 (1968) 397–416
226 W Stober, A Berner, and R Blaschke, The aerodynamic diameter of aggregates of uniform spheres *J Colloid Interface Sci* 29 (1969) 710–719
227 N A Fuks, I B Stechkina, and V A Staroselsky, Determination of distribution of aerosol particles according to size by diffusion method, *AI Trans 201* Translated for Atomics International, Canoga Park, Calif, from Inzy-Fiz Zh Akad Nauk Belorussk SSR, 5 No 12, pp 100–103, 1962
228 J S Townsend, The diffusion of ions into gases *Phil Trans Roy Soc London Ser A* 193 (1900) 129–158
229 Wendell DeMarcus and Jess W Thomas Theory of a Diffusion Battery, Protective Equipment Evaluation Program, ORNL-1413 Oak Ridge National Laboratory, Oak Ridge, Tennessee, 1952
230 Jess W Thomas, The diffusion battery method for aerosol particle size determination *J Colloid Sci* 10 (1955) 246–255
231 L W Pollak and A L Metnieks, On the determination of the diffusion coefficient of heterogeneous aerosols by the dynamic method *Geofis Pura Appl* 37 (1957) 183–190
232 *Symposium on New Methods for Particle Size Determination in the Subsieve Range* Washington, D C, March 4 1941, Am Soc Testing Mater Spec Tech Publ No 51, 1941
233 *Symposium on Particle Size Measurement* Boston, Mass, June 26 27, 1958, Am Soc Testing Mater Spec Tech Publ, No 234, 1958
234 *The Physics of Particle Size Analysis Brit J Appl Phys Suppl 3, London* The Institute of Physics 1954
235 *Symposium on Particle Size Analysis Trans Inst Chem. Eng Suppl 25* 1947
236 G Herdan, *Small Particle Statistics* Elsevier Publishing Co, Amsterdam, Netherlands, 1953
237 Riyad R Irani and Clayton F Callis, *Particle Size Measurement Interpretation and Application* John Wiley and Sons, Inc, New York, N Y, 1963
238 Geoffrey Martin, Chas E Blyth, and Harold Tongue Researches on the theory of fine grinding Part I Law governing the connection between the number of particles and their diameters in grinding crushed sand *Trans Ceram. Soc*, 23 (1924) 61–118
239 Philip Drinker, The size-frequency and identification of certain phagocytosed dusts *J Ind Hyg* 7 (1925) 305–316
240 Theodore Hatch, Determination of "average particle size" from the screen analysis of non-uniform particulate substances *J Franklin Inst*, 210 (1933) 27–37
241 Paul S Roller, Law of size distribution and statistical description of particulate materials *J Franklin Inst*, 223 (1937) 609–633
242 H E Schweyer, Particle size studies, methods *Ind Eng Chem.* 14 (1942) 622–632
243 F Kottler, The distribution of particle sizes, Part I, *J Franklin Inst* 250 (1950) 339–356
244 F Kottler, The distribution of particle sizes, Part II, *J Franklin Inst* 250 (1950) 419–441
245 F Kottler, The goodness of fit and the distribution of particle sizes, Part I, *J Franklin Inst*, 251 (1951) 499–514
246 F Kottler, The goodness of fit and the distribution of particle sizes, Part II *J Franklin Inst*, 251 (1951) 617–641
247 J R Ashford, Some statistical aspects of dust counting *Brit J Appl Phys* 11 (1960) 13–21
248 W Stober, H J Witt, and M Arnold, Teilchengrossenmessungen an anorganischen und biologischen Partikeln *Zeiss Mitt* 2 (1962) 281 308

249. C. N. Davies, Measurement of particles. *Nature,* 195 (1962) 768–770.
250. W. Stöber, Statistical size distribution analysis. *Lab. Invest.,* 14 (1965) 154–170.
251. Harry J. Ettinger and Samuel Posner, Evaluation of particle sizing and aerosol sampling techniques, *Am. Ind. Hyg. Assoc. J.* 26 (1965) 17–25.
252. Othmar Preining, On the evaluation of aerosol size distributions, *J. Rech. Atmospheriques* 2 (1966) 145–151.
253. J. Cartwright, Particle shape factors, *Ann. Occupational Hyg.* 5 (1962) 163–171.
254. J. Raymond Hodkinson, The effect of particle shape on measures for the size and concentration of suspended and settled particles, *Am. Ind. Hyg. Assoc. J.* 26 (1965) 64–71.
255. C. N. Davies, Size, area, volume and weight of dust particles. *Ann. Occupational Hyg.* 3 (1961) 219–225.
256. E. Cunningham, On the velocity of steady fall of spherical particles through fluid medium, *Proc. Roy. Soc.* (*London*) Ser. A, 39 (1910) 357–365.
257. N. A. Fuchs, *The Mechanics of Aerosols,* Rev. ed., MacMillan Co., New York, 1964.

DISCUSSION

D. Craig: I would like to know what particle sizes you can generate from the Fulwyler generator.

O. G. Raabe: The particles that we've generated and have shown here are about 3 μ. The droplets we've been able to produce with this device have been around 25 μ in diameter. Of course, the resultant particle size after evaporation of the water and after chemical effects will depend upon the chemical nature and the concentration of material that you have. Surely, particles as small as half a micron can be produced without too much trouble – and of course this becomes related to the droplet size and depends on the amount of impurities that one may have in the droplet.

Craig: How much below 0.5 μ can you go?

Raabe: I don't know. We haven't gone much smaller than 1 or 2 μ. That's the smallest we've made, but we haven't attempted to make them smaller as yet.

Craig: And the concentrations?

Raabe: This device yields about 100,000 particles per second. We can quickly calculate that 10^5 particles per second, and a liter per second of dilution air would be 100 particles per cc, and that would be 60 liters per minute of aerosol. This would be quite acceptable and yield 100 particles per cc.

Craig: The other comment concerns the very complicated use of mass median diameter and σ_g. You showed one example where, with a σ_g of 2, a 1-μ CMD gave you a 4.2-μ MMD. Now if you go to a σ_g of 2.4, the ratio is 1 to 10, and if you go to 3.5, it is much greater. So one questions whether there is any value at all in these relationships when the σ_g gets above, let's say, 2.4. I wonder if you have any comment on this.

Raabe: Well, I think that most investigators would prefer not to have σ_g's much bigger than 2, and I'm sure the nebulizers people choose have geometric standard deviations that are smaller than 2. Of course, with the Fulwyler device the geometric standard deviation is less than 1.01.

Craig: Yes, but these are artificially generated aerosols. In practice, in the real world, one often gets aerosols that have a much greater standard deviation. At least, I have observed this.

Raabe: I think the only question then is whether the distribution function that you are using is satisfactorily representative of the particles that you are dealing with; and if it is, then it is still quite usable.

J. Kleinerman: Dr. Raabe, which of these various particle generators have denaturation effects on organic or protein materials?

Raabe: If I understand you, the question concerns denaturation of proteins. The ultrasonic generators could produce certain types of denaturation because of the tremendous amounts of concentrated energy. Ultrasonic nebulizers have very large evaporative losses because they actually heat up the liquid considerably; and at the peak of the geyser where droplets are being literally shaken off, there are quite a few molecular vibrations that are going on which could possibly have denaturation effects and can change the chemical states of some organic chemicals.

P. E. Morrow: Another question about the Fulwyler generator. Has a detailed description of its construction and performance characteristics been published?

Raabe: The Fulwyler droplet generator and another generator designed by Strom in Sweden, which works on basically the same principle, have both been described in the literature. Fulwyler described his generator as a cell separator in *Science* in 1965; and in January of this year, in *Review of Scientific Instruments,* he described it in great detail. Strom has also described his generator – which, instead of an ultrasonic transducer, uses an orifice plate (with electroconstrictive elements) to break-up the jet. It works on basically the same principle as the Fulwyler generator, and Strom indicated that with the device he could produce droplets 15 μ or so in size. He also got extremely good uniformity of final particles. That article was published in June of this year in *Review of Scientific Instruments.*

H. L. Berke: Otto, you made a calculation of aerodynamic size and real size, and I notice that you have a density factor there. Would you care to comment on the relationship of the real density and the density we find in particles that are generated, by any method? In other words, could you give us a comparison of bulk density and particle density.

Raabe: Yes. The density one finds in the handbook for a particular material may not be the density of the particles that are produced. It might be if, by chance, the particles were passed through a, say, heated column that was above the melting point of the material. Then we might get very dense particles which had exactly the density the handbook indicates for the bulk material.

However, in most cases of real aerosol generation the density of the particles is somewhat less than the theoretical density; and this may be related to conditions of drying, in the case of soluble materials. And it may also be related, in the case of insoluble materials, just to the conditions by which the particles are formed.

Particles of insoluble materials formed by the technique of Kanapilly and co-workers for degrading organic chelates have densities of approximately one-half the density of bulk material.

CHAMBER DEVELOPMENT AND AEROSOL DISPERSION

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ABSTRACT

Most bioassays with suspected carcinogenic inhalants are carried out by passive inhalation In these studies small mammals are exposed, in inhalation chambers, to fresh tobacco smoke, urban air, diluted automobile exhaust, synthetic air pollutants, occupational respiratory environments, or pure chemicals Although each test requires a specific design, several uniform problems tend to occur during passive inhalation These include particulate aggregation, physical and chemical aging, formation of concentration zones, and increased toxicity with time-delayed exposure to short-term inhalants Since it represents the chemically most complex and densest man made respiratory environment, tobacco smoke is particularly affected by these problems Progress with inhalation chambers used in studying cigarette smoke will be reviewed For anatomic and physiologic reasons, it is doubtful whether enough tobacco smoke can enter the lower respiratory tract to elicit a neoplastic change, although the upper air passages may receive sufficient smoke exposure for such a response

Bioassays of carcinogenic inhalants can be carried out by direct application of the gas, vapor, or aerosol to the respiratory system, or by exposing the animals to a carcinogenic respiratory environment in inhalation chambers In tobacco carcinogenesis we call the first type of experiment active inhalation, and the second type passive inhalation

In our studies at the Sloan-Kettering Institute our major objectives in environmental respiratory carcinogenesis have been the identification of carcinogens, tumor promoters, tumor initators, the determination of their origin, the study of the routes and mechanisms for their formation and, finally, the reduction of these toxic agents in our environment [1,2] We had already realized at the onset of our investigations that postponement of inhalation experiments meant temporary neglect of certain important aspects of respiratory carcinogenesis Since urban as well as personal pollutants are relatively weak

carcinogens, the target organs of the test animals have to be exposed to relatively high doses of the aerosols. Simple experiments with ^{14}C-labeled nicotine as cigarette tobacco additive showed that in passive inhalations relatively few aerosol particles will reach the lower respiratory tract of the animals, so we could not expect to induce tumors in small mammals in this manner. Ten to fifteen years ago little was known about toxic and tumorigenic agents in tobacco smoke and urban air, and knowledge of the physicochemical conditions prevailing in these aerosols was scarce.[3,4] During recent years, however, intensive investigations of the respiratory environments and technical advancements have placed us in a position to test, with a reasonable hope of success, possibly unaltered carcinogenic aerosols by passive inhalation. Furthermore, it appears that passive inhalation experiments can now be designed in such a way that one can compare the activities of weakly carcinogenic aerosols. Carcinogenic bioassays of inhalants found in the general environment, with active inhalations and large mammals, are both costly and time consuming. This applies to voluntary active inhalations by trained monkeys, as studied by Jarvik,[5] as well as to forced active inhalations by dogs via a tracheotomy, as studied by Auerbach and Rockey.[6,7] On the other hand, these active inhalations can effectively show the physiological and pathological changes and their sequences as they occur in mammals exposed to weakly carcinogenic respiratory environments. Such experiments may also lead to the induction of bronchogenic carcinomas that are similar in histological type to those seen predominately in man. Since in tobacco studies it is not feasible to fully duplicate the human setting, we regard both passive and active inhalation experiments as complimentary and essential in the study of respiratory carcinogenesis.

FORMATION AND AGING OF ENVIRONMENTAL AEROSOLS

A significant amount (and often all) of the aerosols in our environment derive from incomplete combustions of organic matter.[1,2] These incomplete combustions determine the chemical composition of aerosols and significantly affect the particle-size distribution in the air we breathe. In general, controlled combustions produce particles in the range of 0.1–10 μ.[1,8] Figure 1 shows the size range of particles found in our atmosphere.[9] The graph depicts as "lung damaging" those particles which range from 0.25–10 μ. Particles of less than 0.25 μ are seldom retained in the lungs, and particles with diameters greater than 10 μ are lodged in the upper respiratory tract and thus do not reach the bronchi.[1,10] It has then been suggested that for physiological and pharmacological studies the term *aerosol* should be limited to airborne dispersates of particles less than 1 μ.[11] In inhalation studies with small rodents the particles have to be kept below 1 μ.[12]

Polluted urban air as inhaled by man is already "aged." (In this part of the discussion we restrict the term *aging* to aggregation of particles.) In passive inhalation experiments with urban air we need to keep temperature and

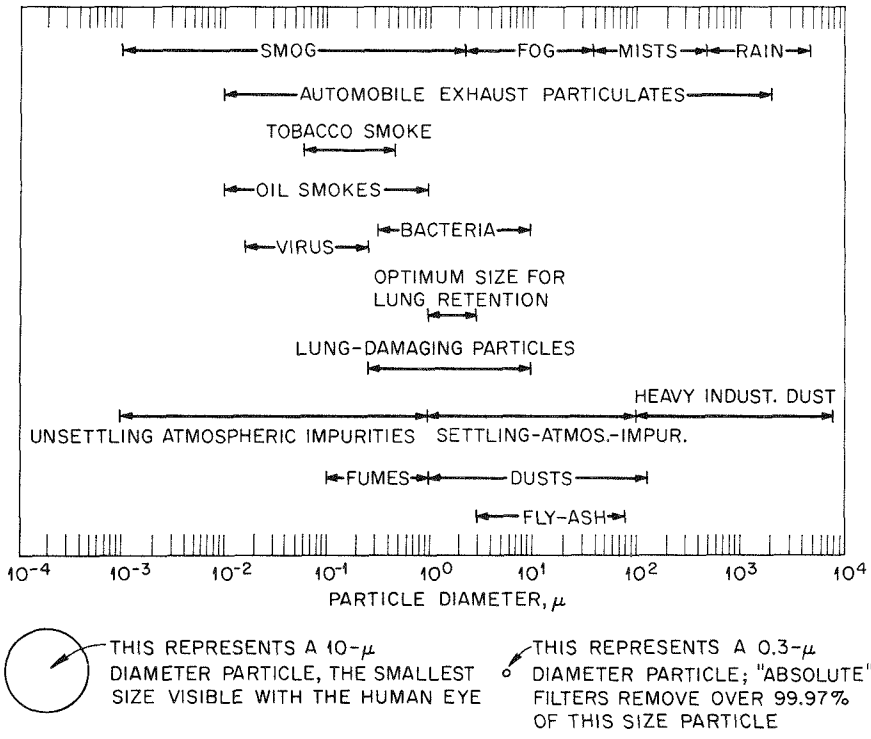

Fig. 1 – Size ranges of particles found in the atmosphere. (Modified from Begeman and Colucci[9])

humidity the same as at the sampling site, since elevation of both factors is reported to increase particle size.[13] To exclude photo-oxidations we should expose the sampled urban air only to light of the spectral range existing at the sampling site. Exposure of sampled pollutants by fluorescent light, as often found in laboratories, should be avoided or reduced to a minimum. Perhaps the best solution is to expose the aerosol only to yellow light during the experiments.

Rather different precautions have to be taken when one exposes animals to freshly generated tobacco smoke. Keith and Derrick[14] demonstrated that the particle size distribution of cigarette smoke changes within a few minutes after formation (Fig. 2). This change, although significant, appears to be rather slow, especially in view of the fact that cigarette mainstream smoke is a highly concentrated aerosol with about 5.3×10^9 spherical droplets per milliliter. The relatively slow change in particle size of tobacco smoke is explained by its small coagulation constant of 3.4×10^{10} ml/sec.[14] In addition to the time-dependent coagulation of cigarette smoke, recovery studies on man suggest that high humidity leads to quick aggregations of smoke particles (Fig. 3).[15]

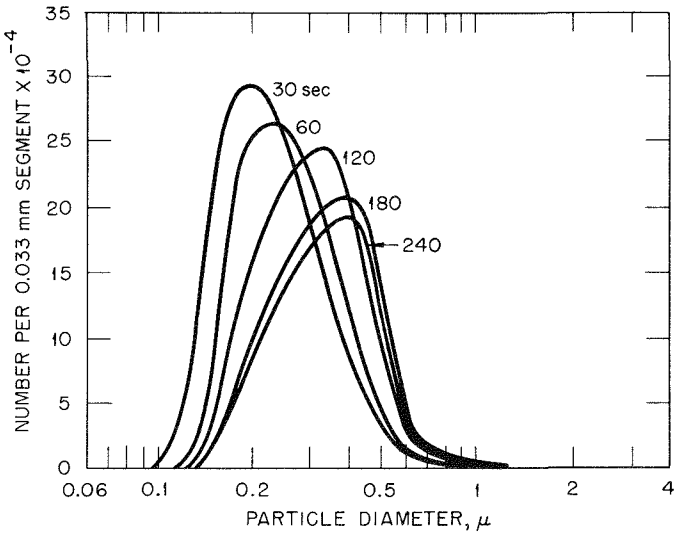

Fig. 2 – The effect of aging on diluted cigarette smoke. (Modified from Keith and Derrick[14])

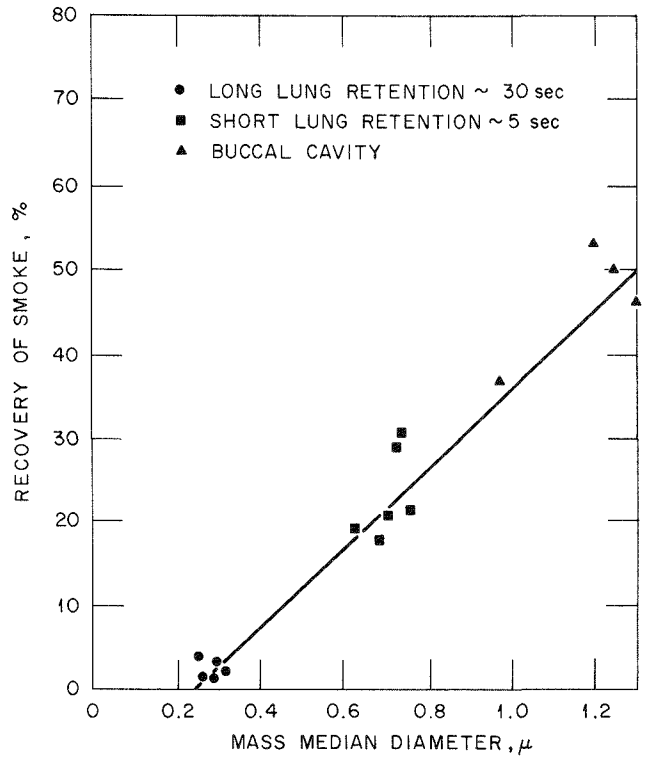

Fig. 3 – Relation of recovery of cigarette smoke from the respiratory tract to mean particle size. (Modified from Mitchell[15])

CHEMICAL CHANGES DURING AGING OF AEROSOLS

For many years it has been suspected that aerosols in polluted air are chemically changed during aging.[16] However, the changes that actually occur remain unexplored, with the possible exception of photo-oxidations and secondary chemical reactions in tobacco smoke. Although we can minimize photo-oxidations by working under yellow light, such precaution has hardly any inhibitory effect on other secondary reactions.

Nitrosamines

The artificial formation of *N*-nitrosamines in cigarette smoke has attracted considerable attention due to the fact that a large number of these compounds are carcinogenic. Two routes are suspected to lead to their formation. These are (1) the reaction of secondary amines with methyl nitrite (Fig. 4). Tobacco smoke contains a rather large number of secondary amines – such as the alkaloids nornicotine, anabasine, and nornicotyrine, the pyrolysis products, pyrrolidine and indole, and such volatile amines as dimethylamine and diethylamine. In a recent review Neurath[17] listed about four-dozen secondary amines in cigarette smoke. Theoretically, about two dozen of these can form carcinogenic *N*-nitrosamines in tobacco smoke. In general, *N*-nitrosamines are carcinogenic to the experimental animal if both alkyl substituents have primary structure ($-CH_2-$), or if one has a primary structure and the other a secondary structure ($\geq CH-$;).[18] Tobacco smoke and most other

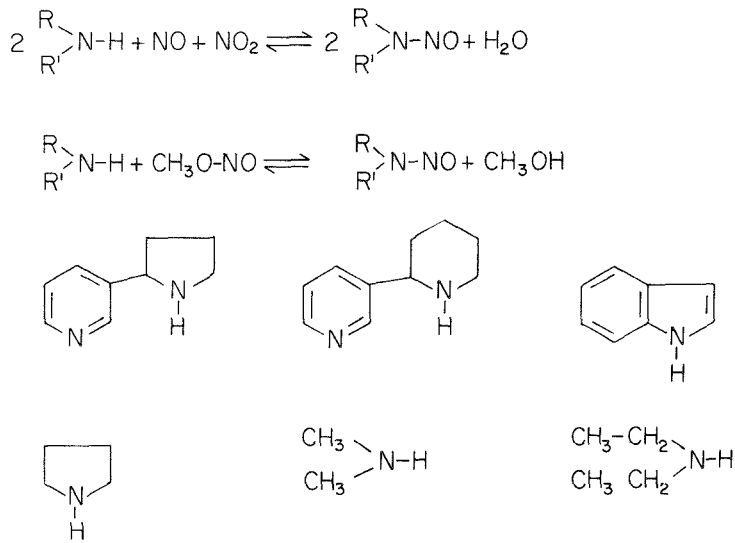

Fig. 4 – Formation of *N*-nitrosamines in the environment.

combustion products of plants contain secondary amines as one group of precursors for *N*-nitrosamines. Under normal conditions, however, it is unlikely that fresh cigarette smoke contains NO_2 or HNO_2. Practically all nitrogen oxides are formed by thermic decomposition of alkali nitrates during puffing. Such nitrates are found in tobacco in concentrations of 0.0–5%.[1] Since the thermic decomposition of alkali nitrates to NO, NO_2, and O_2 occurs at temperatures above 450°C, the decomposition must happen in the burning cone. The atmosphere in the burning cone, which includes about 8 volume percent of hydrogen, is a reducing atmosphere; therefore, the thermically generated and activated NO_2 is either reduced or reacts as scavenger with organic radicals.[19] Thus, it is no surprise that in the last puffs of a cigarette Barkemeyer and Seehofer[20] did not find NO_2, and Norman and Keith[21] only traces of NO_2. It takes about 500 sec for half the NO in tobacco smoke to oxidize to NO_2,[22] a fact which strongly suggests that NO_2, and with it some carcinogenic *N*-nitrosamines, can be formed during aging of aerosols in inhalation chambers.

The second possibility for the formation of *N*-nitrosamines lies in the reaction of secondary amines and methyl nitrite. Although cigarette smoke was first reported to contain up to 500 μg methyl nitrite[23] and, according to a recent study, contains up to 100 μg per cigarette,[24] its formation appears to be artifactual. Neurath[17] estimates the concentration of *N*-nitrosamines at 4 ng per cigarette, an amount unlikely to be of biological significance. However, in passive inhalation experiments the artifactual formation of carcinogenic *N*-nitrosamines could introduce an error into bioassays of combustion products. In fact, we suspect that some of the results obtained with aged smoke are based on artifacts. Dontenwill and our group[1,25] reported a few cases of papillomas in the trachea of hamsters which were exposed five times a week for at least 1 year to an atmosphere of diluted cigarette smoke. These tracheal papillomas were microscopically and macroscopically comparable with those induced by diethylnitrosamine (DENA). A rough estimation suggests that the observed incidence rate with aged cigarette smoke corresponds to the tumor yield observed with 1 mg DENA. These tumors, however, are histologically not comparable with the types of lung cancer in man that are associated with cigarette smoking. We also need to consider that *N*-nitrosamines have so far not been reported to be carcinogenic to man.[18]

Organic Radicals

The aging of a combustion product leads to a significant decrease of the concentration of unstable and metastable organic radicals. These two species of radicals are regarded by some investigators to be potential carcinogens.[26–28] Although a recent study by Peacock and Spence[29] suggests that free radicals do not induce tumors in the lungs of mice, we cannot at present regard this report as a final proof for the nonactivity of radicals. For such experiments, rats or hamsters would be the best animals to use, and pyrolytically formed organic

radicals the best agent. Thus, our current knowledge of pyrolytically formed organic radicals does not exclude the possibility that these unstable agents play a role in experimental respiratory carcinogenesis. The existence of unstable organic radicals is limited to a few seconds, again suggesting that during passive inhalations the aging of pyrolytically formed aerosols must be avoided or reduced to a minimum.

Cyanohydrin Formation

Experiments by Nall[30] have shown that in aerosols volatile aldehydes and hydrogen cyanide can form cyanohydrins (Fig. 5). Both volatile aldehydes and hydrogen cyanide inhibit cilia movement. Thus, the biological testing of a freshly generated aerosol should occur immediately and within the closest possible distance between the source of the aerosol and the exposure site.

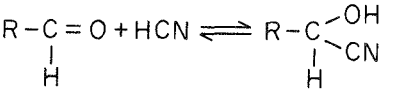

Fig. 5 – Cyanohydrin formation.

Although there is no evidence to indicate that the gas phase of combustion products is a carcinogen, we should not accept as conclusive negative inhalation experiments with the aerosol portion of cigarette smoke that has passed through a Cambridge filter.[31] Filters may contribute to the formation of cyanohydrins and may trap such hydrophilic volatile agents as low-boiling carboxylic acids and phenols.[1] Furthermore, filtration may change the physicochemical equilibria between the gaseous and particulate phases of several other biologically active components, including nicotine and such carcinogens as polonium-210, nickel tetracarbonyl, and arsenic.[1]

TUMORIGENIC AND CILIA TOXIC AGENTS IN NONOCCUPATIONAL RESPIRATORY ENVIRONMENTS

At the present time our knowledge of the nature and concentration of tumorigenic agents in urban and personal air pollution is based only on studies of the particulate matter. Possible exceptions may be model studies with oxonized gasoline by Kotin and Wiseley[32] and experiments with peroxyacetyl nitrate.[33] Tables 1–3 list the carcinogens and suspected carcinogens, tumor initiators, tumor promoters, and cilia toxic agents which have been identified in urban air and tobacco smoke. For the present discussion we have added remarks that relate to the stability and reactivity of these toxic agents.

TABLE 1

Carcinogens and Tumor Initiators in Nonoccupational Respiratory Environments

Components	Cigarette smoke		Urban air		Secondary reactions
	Presence	Relative importance	Presence	Relative importance	
PAH(C In) Benzo[*a*]pyrene Benzo[*j*]fluoranthene Benz[*a*]anthracene	+	In +++	+	In +++	Photo-oxidations
Heterocyclics (C, In) Dibenz[*a j*]acridine Dibenzo[*c,g*]carbazole	+	In ?	+	–	
N-Alkyl heterocyclics (In) 1 Methylindole	+	+	?	?	Oxidation, chemical reactivity
N Nitrosamines (C) Dimethylnitrosamine Nitrosonornicotine	Trace	C?	+?	?	Formed by artifacts
Bladder carcinogens (C) 2-Naphthylamine 4-Nitrodiphenyl	+	–			
Nitro-olefines (C)	?		+	C?	Unstable
Oxygenated hydrocarbons (C)					
Epoxides	–	–	+	C?	Unstable
Peroxides	+	?	+	C?	
Lactones	+	–	+	–	
Radio-isotopes (C) Polonium-210	+	In ??	+	?	
Organic radicals (C?)	+	?	+	?	Very unstable
Metallic constituents (C)					
Nickel tetra carbonyl	+	C?	–	–	Unstable
Arsenic	+	–	+?	–	
Pesticides (C)					
Maleic hydrazide	+	?	–	–	

C, carcinogen, In , tumor initiator

TABLE 2

Tumor Promoters in Nonoccupational Respiratory Environments

Components	Cigarette smoke		Urban air		Secondary reactions
	Presence	Relative importance	Presence	Relative importance	
Volatile phenols Phenol Cresols	+	+	+	(+)	Oxidation
Nonvolatile fatty acids Stearic acid Oleic acid	+	+	+	(+)	Oxidation
N-Alkyl heterocyclics 9-Methylcarbazole	+	+	+	(+)	
Peroxides, epoxides	?	?	+	+	Decomposition
Polymers (Structures unknown)	+	+	–	–	

TABLE 3

Cilia Movement Inhibitors in Nonoccupational Respiratory Environments

Components	Cigarette smoke		Urban air (presence)	Secondary reactions
	Presence	Relative importance		
Volatile aldehydes				
Formaldehyde	+	++	+	Cyanohydrin addition
Acetaldehyde	+	(+)	+	Condensations
Acrolein	+	+++	+	
Hydrogen cyanide	+	+++	+	Cyanohydrin addition
Nitrogen oxides	+	+	+	High chemical activity
Volatile acids				
Formic acid	+	++	+	Increased by ester hydrolysis
Acetic acid	+	+	+	
Benzoic acid	+	–	+	
Volatile phenols				
Phenol	+	+	+	Oxidation
Cresol	+	(+)		

INHALATION CHAMBERS

Between 1952 and 1957 Essenberg[34,35] reported the first extensive passive inhalation experiments. Up to 600 mice were placed in wire-mesh cages which were then transferred into inhalation chambers (Fig. 6). Cigarette smoke was

Fig. 6 – Smoking chamber as used by the Leuchtenbergers. (From Leuchtenberger and Leuchtenberger[36])

drawn through the chamber by a vacuum pump. The cigarettes were on a turntable on the other side of the inhalation chamber. The mice were exposed to the mainstream smoke of up to 1,600 cigarettes five times a week for 6–23 months. Depending on the degree of smoke dilution and the CO concentration of the tobacco aerosol (1–5%), the exposure time for each inhalation was limited to a maximum of 40 min. Additional toxicity may have derived from the volatilized portion of the nicotine, which accounts for about 10% of the total nicotine in the mainstream smoke of blended cigarettes made in the U.S.[1] The incidences of hyperplasia and metaplasia in the bronchi of the smoke-exposed mice were reversible upon cessation of smoking. Our studies have suggested that these changes were secondary to inflammation.[37] Purvis and Ehrlich[38] noted increased susceptibility to inflammation when mice were exposed to relatively low concentrations of NO_2 and some volatile aldehydes. Although the Leuchtenbergers[39] found some alveologenic types of tumors in the exposed mice, they questioned whether these tumors were directly related to cigarette smoke. In retrospect, the rather negative results from these first types of passive inhalations should have been expected. Only a small portion of the weakly carcinogenic aerosol particles becomes a part of the air that is inhaled by the mice. The time lapse between smoke generation and inhalation of the aerosols must have also significantly altered the particle size distribution. Furthermore, one has to realize that these mammals are obligatory nose breathers and thus have intricately developed nasal passages. The smoke irritants also stimulate the exudation of fluids from the nasal passage, thus enhancing further the defensive character of the upper respiratory system of these animals.[37] It has been proven that these animals tend to reduce their breathing pattern when exposed to a toxic respiratory environment.

In 1964 Dontenwill[25] reported on passive inhalation experiments with hamsters in which a set-up comparable to the one just discussed was used (see Fig. 1 in Dontenwill, these *Proceedings*, p. 390). By its use of hamsters this study recognized a new aspect for inhalation studies with small mammals. Experiments with DENA by Dontenwill and Mohr,[40] and with the intratracheal instillation of benzo[*a*]pyrene on a carrier by Saffiotti *et al.*,[41] demonstrated a relatively high susceptibility of the tracheobronchial epithelium of hamsters to carcinogens and a low susceptibility to infection. However, the high rate of dilution of the tobacco smoke and its "aging" in this set-up prevented sufficient exposure for the possible induction of lung carcinomas. Insufficient exposure in such passive inhalation experiments is compounded when groups of hamsters are treated jointly and bury their noses in each others fur.

An advance in the development of inhalation chambers can be seen in a device designed specifically for hamster experiments (Fig. 7).[1] Up to 36 animals

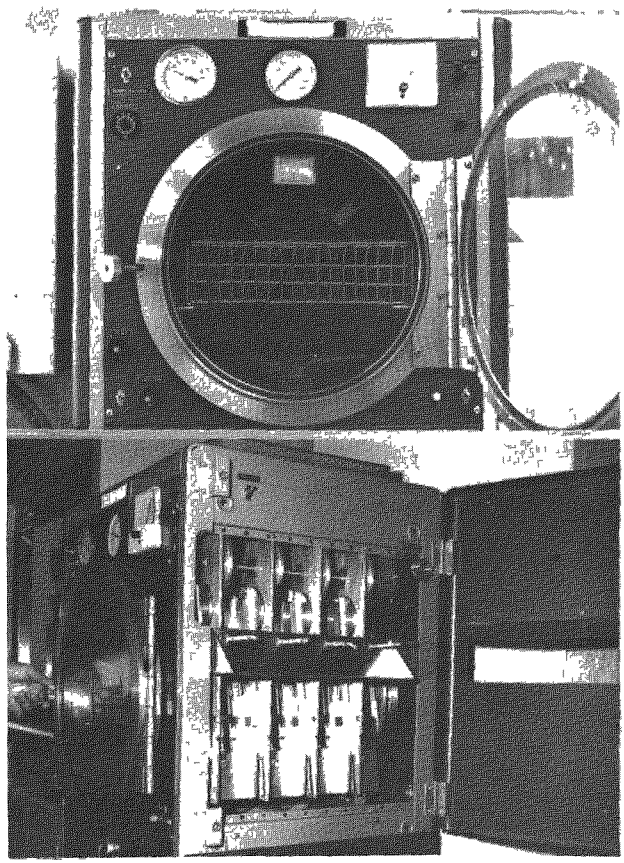

Fig. 7 – Inhalation chamber as used at the Sloan-Kettering Institute. (From Wynder and Hoffmann[1] by permission of Academic Press, Inc.)

can be placed separately into a 70-liter exposure chamber. This compartment has controlled temperature and humidity, a fan for even distribution of the aerosols, and its own piston-type cigarette-smoking machine. The passage between piston and exposure chamber is less than 3 cm long. The actual inhalation experiment begins with a gradual reduction of pressure within the chamber to about 600 mm Hg; the freshly generated smoke of 16 cigarettes is then blown into the chamber. This technique, which leads to an environment with a smoke to air ratio of 1:10, is used with both hamsters and mice. The animals remain in the chamber for 40–50 min per exposure. Exposure of C57BL male mice for up to 20 months revealed only reversible hyperplasia, metaplasia, and a high incidence of infection. Similar bronchial infections occurred in mice exposed only to that portion of cigarette mainstream smoke which passes through a Cambridge filter. The incidence rate of adenomas was comparable to that among control mice. Single and repeated intranasal inoculations of mice with Swine influenza virus had hyperplastic and metaplastic effects. These effects, however, could not be propagated by subsequent exposure to smoke. These observations are similar to those reported earlier by the Leuchtenbergers.[39] Inhalation experiments with hamsters which were carried out for 12 to 18 months led to a few tracheal papillomas. There were no other significant irreversible changes in the trachea or bronchi, a result in agreement with earlier observations by Dontenwill.[25]

Holland *et al.*[42] have developed a device for passive inhalation studies with rabbits (Fig. 8). The animals are tightly packed into individual boxes and exposed to the mainstream smoke of 20 cigarettes daily for as long as $5\frac{1}{2}$ years. In this setting the cigarette smoke passes the rabbit's head for a few seconds once every minute. It is possible, however, for the animal to adopt a breathing pattern that avoids inhalation of the concentrated smoke. Thus, it is doubtful that the exposure is significant. The investigators observed only focal and general hyperplasia in the bronchus of the exposed rabbits, and only 1 out of 30 rabbits developed squamous metaplasia.

More sophisticated inhalation chambers have been introduced during the past few years. One of these is a device used by Harris and Negroni,[43] which was developed from the Henderson machine for the exposure of animals to aerosols of bacteria (Fig. 9). Cigarette mainstream smoke generated by a smoking machine under standardized conditions is diluted with air 1:39 and blown through a plastic manifold distributor. This distributor has 10 openings on each side for holding the animals' noses. The mice are confined in metal boxes and exposed to the diluted smoke of 12 cigarettes for 12 min every other day for the duration of their lives. Upon autopsy the exposed mice showed emphysema and focal hyperplasia but no hyperplastic lesions. Only 1 out of 200 of the exposed mice had a lesion of 1–2 mm diameter. This lesion was similar to a squamous cell carcinoma but failed to be transplantable. Adenocarcinomas, which were not observed in the control mice, occurred in 4% of the mice.

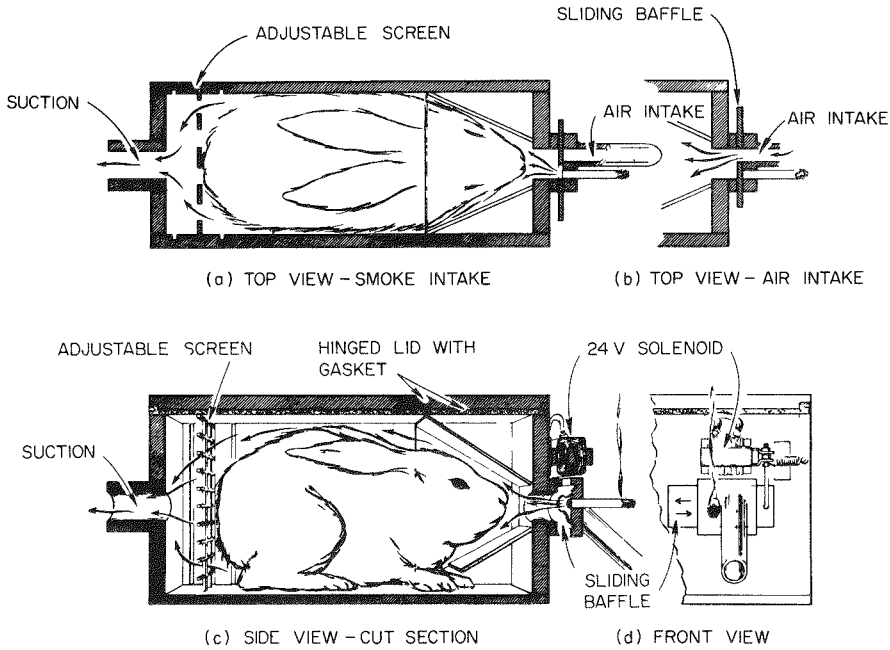

Fig 8 – Individual unit of inhalation chamber for rabbits as used by Holland *et al* (From Holland *et al* [42])

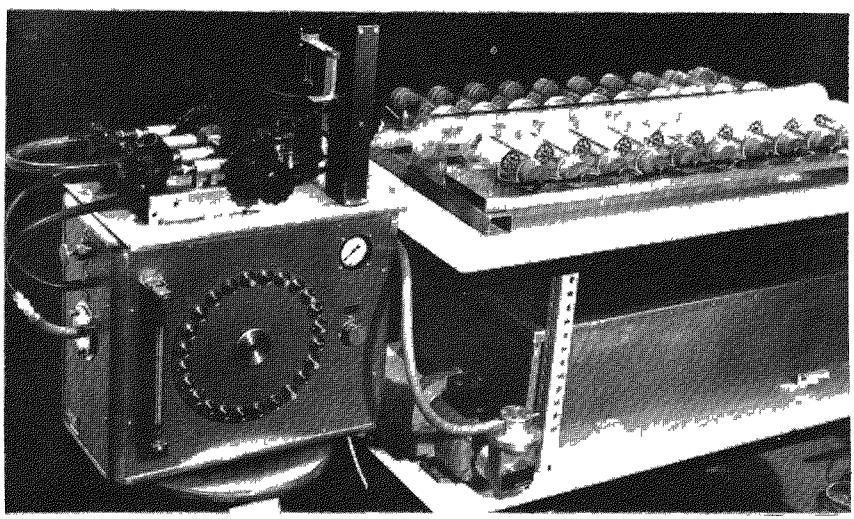

Fig 9 – Harris and Negroni machine for inhalation studies (From R J C Harris and G Negroni, in *Lung Tumours in Animals* pp 497–512, L Severi (ed) Division of Cancer Research, Perugia, 1966)

Another more recent device is the one developed by Dontenwill *et al.*[44] (see Fig. 6 in Dontenwill, these *Proceedings*, p. 393), in which hamsters are placed in individual, exchangeable smoke tubes. The cigarette smoke is generated by two piston-like smoke chambers and diluted with air from 1:4 to 1:8. The diluted smoke is sent into the center smoke chamber and then into the smoke tubes containing the hamsters. Using this device the authors report that they can expose the hamsters to a smoke-air mixture of 1:6.5 three times every 22 min. This study incorporates two essential factors in passive inhalation experiments with tobacco smoke: exposure to smoke that is diluted only 4–8 times, and use of the hamster as a test animal. The rather short distance the smoke has to travel between piston and inhalation chambers appears to be another advantage over the Harris and Negroni smoking device.

The latest machine for passive inhalation experiments was developed by Homburger and co-workers[45] for the American Tobacco Industry Research Council (Fig. 10). When a puff is taken from one to three cigarettes, a cylindrical plunger descends down over the cigarette and pushes the 35 ml of smoke into the central smoke chamber. The distance the smoke has to travel to get into the

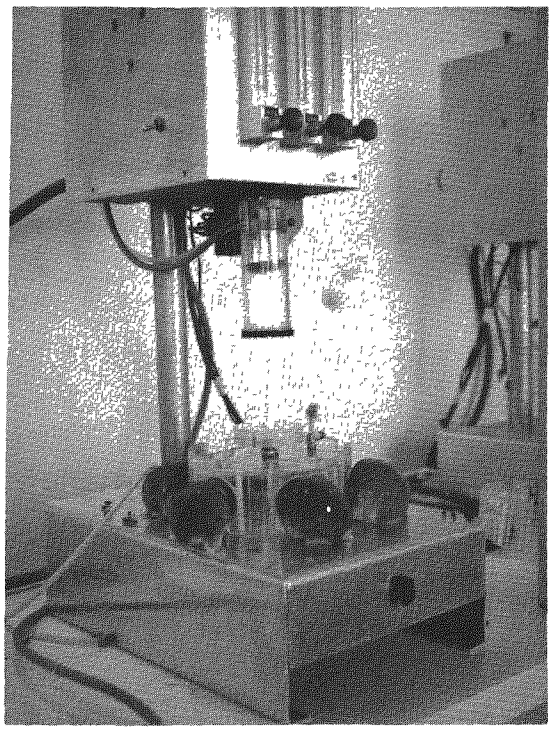

Fig. 10 – "Smoking" machine and inhalation chamber for hamsters as used by Homburger *et al.* (From Homburger *et al.*[45])

inhalation chamber is the shortest of all known systems. After a few seconds the smoke is exchanged for air. This cycle repeats once a minute. The mice or hamsters wear permanent felt collars covered with a light aluminum shield, which does not interfere with their normal life. When smoking exposures are desired the animals are simply plugged into the machine, with only their heads exposed to smoke. A unique feature introduced by this technique is the closing of the noses of the mice or hamsters with a soft rubber band during the smoking. Although no technical details or experiences with this smoke chamber have been reported, it appears that this set up, including the closing of the noses, may lead to promising results for the induction of precancerous changes in the respiratory systems of hamsters.

These last two smoking devices – the ones by Dontenwill and Homburger – or perhaps a combination of both, represent to our knowledge the most advanced designs for inhalation chambers. These machines, with possible further improvements, should also be useful for the bioassay of other freshly generated aerosols by passive inhalations.

ACKNOWLEDGEMENTS

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REFERENCES

1. E. L. Wynder and D. Hoffmann, *Tobacco and Tobacco Smoke, Studies in Experimental Carcinogenesis.* Academic Press, New York, 1967.
2. D. Hoffmann and E. L. Wynder, Chemical analysis and carcinogenic bioassays of organic particulate pollutants. In, *Air Pollution,* Vol. II, 2nd ed, pp. 187–242, A. C. Stern (ed.). Academic Press, New York, 1968.
3. E. L. Wynder, *The Biologic Effects of Tobacco.* Little, Brown and Company, Boston, 1955.
4. *Air Pollution,* Vol. I, A. C. Stern (ed.). Academic Press, New York, 1962.
5. M. E. Jarvik, Tobacco smoking and monkeys. *Ann. N.Y. Acad. Sci.*, Art. 1, 142, (1967) 280–294.
6. O. Auerbach, E. C. Hammond, D. Kirman, and L. Garfinkel, Emphysema produced in dogs by cigarette smoke. *J. Am. Med. Assoc.*, 199 (1967) 241–246.
7. E. E. Rockey, Evolution of cigarette smoking techniques in dogs. *J. Intern. Coll. Surgeons,* 11 (1966) 409–413.
8. P. M. Giever, Analysis of number and size of particulate pollutants. In, *Air Pollution,* Vol. II, 2nd ed., pp. 249–280, A. C. Stern (ed.). Academic Press, New York, 1968.
9. C. R. Begeman and J. M. Colucci, Apparatus for determining the contribution of the automobile to the benzene-soluble organic matter. *Natl. Cancer Inst. Monograph,* No. 9 (1962) 17–57.
10. T. F. Hatch and P. Gross, *Pulmonary Deposition and Retention of Inhaled Aerosols.* Academic Press, New York, 1964.
11. L. Dautrebande, *Microaerosols.* Academic Press, New York, 1962.
12. J. C. Arcos, M. F. Argus, and G. Wolf, *Chemical Induction of Cancer,* Vol. I. Academic Press, New York, 1968.

13 J V Jemski and G B Phillips, Aerosol challenge of animals In, *Methods of Animal Experimentation,* Vol I, pp 273 241, W I Gay (ed) Academic Press New York, 1965
14 C H Keith and J C Derrick, Measurement of the particle size distribution and concentration of cigarette smoke by centrifuge *J Colloid Sci ,* 15 (1960) 340 356
15 R I Mitchell, Controlled measurements of smoke particle retention in the respiratory tract *Am Rev Respirat Diseases,* 85 (1962) 526–533
16 C N Davies, *Aerosol Science* Academic Press, New York, 1966
17 G Neurath, Stickstoffverbindungen des Tabakrauches *Arzneimittel Forsch ,* 19 (1969) 1093–1106
18 P N Magee and J M Barnes, Carcinogenic nitroso compounds *Advan Cancer Res ,* 10 (1966) 163–246
19 D Hoffmann and E L Wynder, Selective reduction of the tumorigenicity of tobacco smoke Experimental approaches *Natl Cancer Inst Monograph,* No 28 (1968) 151–172
20 H Barkemeyer amd F Seehofer, Zur Untersuchung der Gas-Dampf-Phase des Cigarettenrauches 2 Mitteilung Zur Bestimmung des Stickstoff Monoxides (NO) aus der Gas-Phase des Cigarettenrauches *Beitr Tabakforsch ,* 4 (1968) 278–282
21 V Norman and C H Keith, Nitrogen oxides in tobacco smoke *Nature,* 205 (1965) 915 916
22 G Neurath, Zur Frage des Vorkommens von N-Nitroso-Verbindungen im Tabakrauch *Experientia,* 23 (1967) 400–404
23 R J Phillips and E J Hackney, The presence of nitrous oxide and methyl nitrite in cigarette smoke and tobacco pyrolysis gases *Tobacco Sci* 3 (1959) 139–143
24 C H Sloan and B J Sublett, Determination of methyl nitrite in cigarette smoke, *Tobacco Sci ,* 11 (1967) 21 24
25 W Dontenwill, Experimentelle Untersuchungen zur Genese des Lungen-Carcinomas *Arzneimittel Forsch ,* 14 (1964) 774 480
26 D J E Ingram, Electron resonance studies of the free radicals produced in tobacco pyrolysis and in other related compounds *Acta Med Scand* 170, Suppl (1961) 43 62
27 H V Boening, Free Radical and Health, Report C-8 Spindletop Research, Lexington, Kentucky, February 1966
28 J T Cooper, W F Forbes, and J C Robinson, Free radicals as possible contributors to tobacco smoke carcinogenesis *Natl Cancer Inst Monograph,* No 28 (1968) 191–197
29 P R Peacock and J B Spence, Incidence of lung tumors in LX mice exposed to (1) free radicals, (2) SO_2 *Brit J Cancer ,* 21 (1967) 606–618
30 J F Nall, Complexed cyanide in collected cigarette smoke Presented at the 20th Tobacco Chemists' Research Conference, November 1–3, 1966, Winston-Salem, North Carolina
31 H Otto and and H Elmenhorst, Experimentelle Untersuchungen zur Tumorinduktion mit der Gasphase des Zigarettenrauches *Z Krebsforsch ,* 70 (1967) 45–47
32 P Kotin and D V Wiseley, Production of lung cancer in mice by inhalation exposure to influenza virus and aerosols of hydrocarbons *Progr Exptl Tumor Res ,* 3 (1963) 186–215
33 H E Stockinger and D L Coffin, Biologic effects of air pollutants In, *Air Pollution,* Vol I, 2nd ed , pp 445–546, A C Stern (ed) Academic Press, New York, 1968
34 J M Essenberg, Cigarette smoke and the incidence of primary neoplasm of the lung in the albino mouse *Science,* 116 (1952) 561–563
35 J M Essenberg, Further study of tumor formation in the lungs of albino mice *Western J Surg Obstet Gynecol ,* 65 (1957) 161–163

36 C Leuchtenberger and R Leuchtenberger, A correlated histological, cytological, and cytochemical study of the tracheobronchial tree and lungs of mice exposed to cigarette smoke. *Acta. Med. Scand.*, 170, Suppl. (1961) 102–115.
37. E. L Wynder, T. Taguchi, V. Baden, and D. Hoffmann, Tobacco carcinogenesis. IX. Effect of cigarette smoke on respiratory tract of mice after passive inhalation. *Cancer,* 21 (1968) 134–153
38. M. Purvis and R. Ehrlich, Effect of atmospheric pollutants on susceptibility to respiratory infections. II. Effect of nitrogen dioxide. *J Infect. Disease.*, 113 (1963) 72–76.
39. C. Leuchtenberger and R. Leuchtenberger, The need of experimental inhalation studies in animals for the problem of the influence of cigarette smoke on the induction of lung cancer. *Z. Praeventivmed.*, 13 (1968) 122–129.
40. W. Dontenwill and U Mohr, Carcinome des Respirationstractus nach Behandlung von Goldhamstern mit Diaethylnitrosamin. *Z Krebsforsch.*, 64 (1961) 305–312
41. U Saffiotti, F Cefis, and L H Kolb, A method for the experimental induction of bronchogenic carcinoma. *Cancer Res.*, 28 (1968) 104–124
42. R H Holland, E. J Kozlowski, and L Booker, The effect of cigarette smoke on the respiratory system of the rabbit. *Cancer,* 16 (1963) 612–615
43. R. J C Harris and G Negroni, Production of lung carcinomas in C57BL mice exposed to a cigarette smoke and air mixture *Brit. J Med.*, 4 (1967) 637–641
44. W. Dontenwill, G. Reckzeh, and L. Stadler, Berauchungsapparatur fuer Laboratoriumstiere. *Beitr. Tabakforsch.*, 4 (1967) 45–49.
45 F Homburger, P Bernfeld, P Bogdonoff, T Kelley, and R. Walton, Inhalation by small animals of fresh cigarette smoke generated by new smoking machine *Toxicol. Appl Pharmacol.*, 10 (1967) 382

DISCUSSION

A. Furst: I would like to lay the nickel problem to rest, finally. There is no question that nickel and all of its compounds are carcinogenic. Many of them, and in particular nickel carbonyl, have been tested by Dr. Hueper's group, by Dr. Sunderman, and by my group. However, there is no nickel carbonyl in cigarette smoke. We have burned about two or three hundred cigarettes and collected combustion products. We have concentrated and tested with a reagent which can detect a spot of 10^{-4} μg of nickel on paper. We could not find any nickel in the mainstream smoke or in the compensates. Nickel can be found sporadically in some cigarettes, and when this happens, the nickel is in the ash.

So, once and for all, I think we can forget the idea that nickel carbonyl is any culprit, or any factor, in cigarette smoke.

D. Hoffmann: If you may recall, the chapter we wrote in our book on nickel carbonyl paralleled your standpoint. Today, however, as analytical chemists, we regard paper chromatography as not very advanced. When you use flame photometry, as reported in an outstanding article in *Analytical Chemistry,* there is nickel present in the mainstream smoke of cigarettes.

Therefore, since we are not the type of people who are not willing to change their concept, we think, today, that cigarette smoke contains nickel as shown by flame photometry, although we do not know in which form it is present. We know only that it's at a very low concentration.

S. Laskin: With reference to equipment problems (they are extraordinarily complex) and the nature of this specific type of problem, I would like to call attention to two areas. One is the equipment design for retention studies in which a face-mask follow system for a rabbit was extremely well designed and did not allow any leakage [see H. E. Stokinger, C. W. LaBelle, and co-workers, Toxicity following inhalation. In, *Pharmacology and Toxicology of Uranium Compounds,* Division VI, Vol. 1, pp. 423–700, C. Voegtlin and H. C. Hodge (eds.). McGraw-Hill Book Company, Inc., New York, 1949.] It followed the respiratory rate of the rabbit and permitted very accurate dosing.

The other area is the number of developments, at the Lovelace Foundation, in exposure of small animals by a variety of techniques.

Hoffmann: I may have omitted quite a lot of designs. I have to apologize, but I have limited myself, as stated, to work done on tobacco smoke and published in the literature.

D. Craig: I don't think we should let go unchallenged one statement that you made quite early regarding the deposition of very fine particles. You stated that no particles less than 0.2 μ will be deposited in the alveolar regions of the lung.

Hoffmann: I did not intend to give the impression that no particles of 0.2 μ and smaller are retained in the lung upon inhalation, but rather that the retention is insignificant.

Craig: How were your measurements made? I would be inclined to think that the distributions you showed for tobacco smoke were very much a function of the method used to obtain those distributions.

R. Rylander: Dr. Hoffmann, in connection with the breathing patterns and the penetration of tobacco in nose-breathing animals, you mentioned some Russian studies stating that changes had not been found after exposure. As far as I can recall, changes were found in the goblet cell pattern. Abnormalities that could be interpreted as precancerous changes were also found in the bronchial tree, together with papillomas. Furthermore, I don't think your statement about the rodent holding its breath when exposed to tobacco smoke can be generalized. It is true that most of the animals will not breathe on the 1st and 2nd, and maybe not even on the 3rd day of exposure; but within a week they will adapt and breathe. It might be with a changed respiratory pattern, but they will definitely breathe. Some of the animals, in long-term experiments, even get used to or addicted to the smoke, so that on their own they crawl up the small exposure tubes and put their noses near the cigarette. Concerning nose breathing in general, the small particle size of tobacco smoke will allow a large amount of the particles to pass through the nose and down into the lungs where they deposit. It's true that some of the water-soluble substances, preferably in the volatile phase, will be absorbed in the nose; but this happens in humans, too, as

we have shown in several experiments. In summary, I don't think that a change of breathing pattern and breathing through the nose is necessarily a very severe drawback in this type of experiment.

Hoffmann: We tried to conduct a study years ago in our laboratory using just the device of Holland *et al.* [*Cancer,* 16 (1963) 612]. We gave one puff a minute, with two seconds per puff. We tried to do it by having the smoking machine on one side of the box and the heads of the animals and the burning cigarettes on the other side. In this setting the rabbits waited just a few seconds until the air did not contain cigarette smoke, or very little, and then they started to breathe again. They just held and did not breathe. Again, our experiments were limited to the device of Holland *et al.*

F. Homburger: I should like to make one comment. I'm very pleased with the compliments you paid us about our machine, but I think that we should also give thought to the animal. We are also now turning to hamsters; the machine has been adapted to them, but we are finding that they are extremely variable, depending on their genetics. Mrs. Barbara Grossman of the Harvard School of Public Health has observed that upon intratracheal instillations there is a very low mortality in one inbred strain, whereas three of the other inbred strains have a high mortality. I should like to caution investigators who are going to work with hamsters that, before making a tremendous investment in a 2-year or longer experiment, they should decide very carefully what kind of hamster they are going to use. The question that has been discussed back and forth for many years for mice, whether to use random-bred or inbred mice, now confronts us with hamsters. We have to make the right decision, or else we might later find that we did the wrong experiment.

B. R. Davis: I would agree with Dr. Hoffmann about the animals tending to hold their breath. We have recently developed a smoking machine for the exposure of rats, and these animals, if exposed intermittently to puffs of smoke and fresh air, after a few months will learn to hold their breath for periods up to 10 sec. We have overcome this problem by simply increasing, to about 5%, the carbon dioxide content in the fresh air used to scavenge the chamber between puffs of smoke. This stimulates the respiratory center sufficiently to prevent the animals from holding their breath, and we can obtain very satisfactory smoke intakes, as indicated by carbon monoxide levels in the blood.

Hoffmann: I think this is a very ingenious idea. I wasn't aware of this technique, but as you said, it is a good way to overcome these changes in the breathing pattern.

GENERATION AND MONITORING OF GASES FOR INHALATION STUDIES

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ABSTRACT

The use of challenge gases in inhalation studies imposes many of the same requirements on the exposure systems as does the use of gases in other types of environmental or even reaction studies These may include gas purity, knowledge of composition, flexibility, constancy of generation and safety Additionally, there may be requirements for protection of the experimental subjects, such as special temperature and humidity conditions, and compatibility with unusual exposure configurations — Methods of test gas generation are reviewed, with consideration given to advantages, limitations, and special application These methods range from the use of pure compressed gases and the gasification of liquids to on site generation by chemical or electrochemical procedures Methods and materials for gas handling are described, including the preferred approaches to injection and mixing Reference is made to differences between large and small systems Special attention is given to the metering of dosing gas and carrier gas (*i e* breathing air) — The problem of dosing gas assay is discussed with emphasis given to currently accepted continuous monitoring practices Factors included are sampling strategy, sample handling, availability and selection of equipment, and data processing

A significant part of any research effort involving the use of physical facilities is the design, operation, and maintenance of such facilities This is no less true for inhalation studies than for other areas of work Here we are concerned with the production of a controlled test atmosphere containing accurately known small quantities of challenge gases (single or multiple component) in breathing air (The term *small quantities* is used to mean the volumetric parts per million range) The problem may be compounded further if foreign aerosols are to be added, but this subject will not be treated in this paper The main objective of providing such mixtures is likely to be complicated by the factors of breathing air composition, reaction of challenge gases with other gases, reaction or adsorption on surfaces (including test animals), and physical integrity of the test system Thus the question of component assay for verification of theoretically

calculated concentrations becomes inextricably intertwined with the major task at hand.

In the design of inhalation facilities one must consider a number of ancillary issues, some of which can assume major proportions in any given situation. These include size of the test chambers; mode of operation (short *vs.* long term, unattended *vs.* attended, intermittent *vs.* continuous); air conditioning; exhaust gas treatment; personnel safety; and use of warning devices.

This paper will review the methods of generating and dispensing test gas, the procedures for introducing the gases to the carrier gas (*i.e.*, breathing air), and the use of continuous analyzers for challenge gas assay. Special problems associated with materials, with corrosive and liquefiable gas, and with chamber size will be treated where applicable.

GENERATION OF TEST GASES

The generation or production of test gases is common to a variety of experimental situations. The end use of the resulting controlled mixtures may be for calibration of analytical methods or instruments, for studies of reaction kinetics, for the exposure of materials, or for bioassay. The controlled atmospheres may be used in a static or dynamic mode. Most uses of controlled atmospheres in exposure testing involve the dynamic mode, since constant replenishment of the active component is necessary to maintain equilibrium conditions.

Still further subdivisions may be made, as has been done by Lodge[1] in his general review of test atmosphere production. He uses the terms *quasidynamic* and *dynamic* to distinguish between systems in which there is an enlargement having most of the volume (quasidynamic) and those which have a substantially linear flow rate throughout the system (dynamic).

Systems for inhalation studies almost always will be classified as quasi-dynamic. The principal significance of this distinction is difference in time response to step changes in the composition of the test gas. If we consider our enlargement or chamber to be an approximation of a thoroughly stirred reactor, the following differential equation will describe the time response to a step concentration change:

$$\frac{dc}{dt} = \frac{C_e - C}{\lambda}$$

where

C = instantaneous concentration at elapsed time (t),

C_e = concentration of new environment at equilibrium,

t = elapsed time after system is exposed to new environment,

λ = time constant (same terms as t)

$$= \frac{V}{Q}$$

where

V = test volume

and

Q = volumetric flow rate.

Upon integration, and adding the term C_o to represent concentration at t = 0, the expression becomes

$$\frac{C_e - C}{C_e - C_o} = e^{-t/\lambda} .$$

Rose and Brandt[2] and Bryan and Blackmore[3] have reported results of chamber experiments in which experimentally determined time constants were compared with those calculated from the above equation. Both report extremely close agreement in results.

The practical consequences of the system time constant are several. Systems with short time constants will exhibit less smoothing of any variations in challenge gas input than will those with longer time constants. In experiments with relatively short exposure times, account must be taken of the time constant to permit proper calculation of the dosage. The initial response of experimental animals to irritating gases may vary with the time constant even though the equilibrium concentrations are the same. Finally, knowledge of chamber blow-down time may be important for personnel safety considerations. A further mathematical treatment of exposure chambers is given by Silver.[4]

The actual physical production of test atmospheres involves both the active component and the carrier gas. In the case of quasidynamic systems, with which we will concern ourselves from this point on, knowledge of the active component concentration must be obtainable by theoretical calculation from mass or volumetric (at known temperature and pressure) flow rates, or by assay techniques. Systems utilizing the first approach are often described as primary, while those using the assay technique are called secondary. As will be seen, the use of both techniques in concert is often desirable.

As a general principle primary systems which use conservative physical properties for rate determination are inherently capable of greater accuracy and reliability than those which do not. A concentration determined by measurements of the active component's weight loss would theoretically have a higher level of confidence than one based on volume.

In general, methods of contaminant addition and flow metering vary in their adherence to fundamental principles, even when conservative properties are not being measured. The possibility of sampling error and change in system characteristics are also related to addition and metering methods. An absolute volumetric measurement should, for example, be more accurate than a flow determination made by a variable orifice meter. Further, a pitot tube

measurement of airflow has a greater likelihood of sampling error than does a total flow measurement with an orifice or venturi meter.

The actual production or generation of test gases is accomplished by a variety of mechanical, physical, or chemical means. An excellent review of gas addition and mixing techniques was recently published by Hersch.[5] This paper will use much the same nomenclature used by him. Hersch reports only on those techniques and apparatus which do not have solid moving parts (other than stopcocks), but few of the several elegant methods, principally calibration, have been used for other than very small flows.

Mechanical Methods

These include methods that positively displace the contaminant gas or move it by pressure gradient. The positive displacement techniques involve, most commonly, a liquid or solid piston in which the motion of the piston is used to displace the gas through a known volume. They are inherently sequential procedures and are rarely connected to a contaminant gas reservoir, thus limiting the applicability for easy long-term exposures. So far as can be determined, liquid pistons have not been used for dispensing gas to exposure chambers, but they do offer the potential advantage of smooth movement and no leaks.[6]

Nelson and Griggs[7] have described a precision, gas-tight syringe technique suitable for use with both gases and volatile liquids. The contaminant gas or liquid is dispensed from a Teflon-tipped syringe having a Teflon needle, with the plunger being driven by a lead screw attached to a synchronous motor that has both a variable-speed gear box and fixed gear reducer. Syringes of .05–20 ml capacity have been used. When used in conjunction with a multiple rotameter set for the carrier gas, contaminant concentrations ranging from .05–2000 ppm have been produced. An accuracy of ±1% is claimed.

A system of somewhat greater airflow capacity has been described by Thomas and Amtower.[8] It has a motor-driven syringe that injects the contaminant gas directly into a blower-supplied airflow. The total volume passes through a calibrated anemometer.

Holl and Mühleisen[9] used a spirometer as a large positive displacement system to deliver hydrogen chloride into a substantial airflow.

One other approximate approach to the positive displacement technique would be the application of peristaltic type pumps. These are commonly used for liquid feed or metering where contamination of the fluid or its corrosiveness is a problem. These pumps operate by the progressive compression of a flexible tubing with fingers, nutating plates, or rollers. Assuming no dimensional change in the tubing, such a device operated by a synchronous motor produces a unit volume each cycle. So far as is known this technique has not been used for the preparation of controlled gas mixtures.

By far the most commonly used technique in the production of controlled gas mixtures involves the use of gas and/or air pressure as a driving force. These

systems commonly use compressed gas sources, thus providing a sufficient reservoir of contaminant gas for extended dynamic operation with large volumes of air Unfortunately, not all materials of interest are available in this form, and some which are exhibit special problems (*e g*, gases with boiling points near ambient temperature)

Those systems operating with pressure as a direct driving force utilize flow restrictors as metering devices These restrictors, which must have a reproduc ible, stable pressure drop-flow relationship over the range of interest, are of two basic types laminar (streamline) flow and turbulent flow

The distinction between the two flow types is based upon the Reynold's number encountered over the range of flow to be used This number is the dimensionless quantity shown below

$$\mathrm{Re} = \frac{\mathrm{Du}\rho}{\mu} \text{ (pipes of circular cross section)}$$

where

D = pipe diameter,

u = linear flow velocity,

ρ = density,

and

μ = viscosity,

all in consistent units

At Reynold's numbers below a critical point, $\mathrm{Re_c}$, flow is laminar The velocity at this point is given as

$$V_c = (\mathrm{Re_c})\frac{\mu}{\mathrm{D}\rho}$$

A generally accepted value for $\mathrm{Re_c}$ is 2100 Since μ and ρ are determined by the gas being dispensed, the only variable is the diameter, D For true laminar-flow elements the principle advantage is that pressure drop is a function of the first power of the flow rate (Poiseuille's law), and calibration curves can be prepared easily and accurately From a practical point of view there are some limitations resulting from maintenance of the restrictor device In order to obtain the low Reynold's number required, D must be very small, such as with a fine-capillary porous plug or a finely crimpled metal mesh bundle Dirt or corrosive gases which effectively change D result in a change in flow factor

Nevertheless, when used properly, the laminar flow approach is useful Saltzman and Wartburg[10] used a semiporous asbestos plug as a flow restrictor in a T-junction, with the flow entering one horizontal arm, the leg of the T submerged in water to a depth necessary to obtain the proper back pressure and to carry away the bulk of the gas through bubble formation, and the desired flow leaving the other horizontal arm of the T, which contains the asbestos plug

J. D. MacEwen,[11] in a discussion of techniques for toxicity studies, indicated the successful use of laminar flow elements made of stainless steel. Because of the relatively narrow flow range possible, serial dilution techniques have been used to increase dynamic range. General experience with such systems has often been that they are subject to pressure perturbations and other anomalous behavior, which results in less than good reproducibility.

The most common pressure-differential metering devices for preparing controlled gas mixtures are variable orifice meters, otherwise known as rotometers. In operation the gas to the metered flows upward through a tapered channel of increasing cross section. A float of known density is lifted to a point at which its weight is equal to the velocity head of the flowing gas. The scale reading at that point is interpreted in terms of a calibration chart that specifies density and viscosity. Rotometers are turbulent flow devices, a class in which pressure drop (or head loss) is a function of the velocity squared. Thus velocity fluctuations may result in severe float oscillation. In the case of very high frequency perturbations the rotometer may operate as a root mean square device, thus making flow interpretation difficult.

As in the case of all metering systems based on pressure drop, the flow-metering valve for the rotometer must account for a substantial portion of the pressure drop in the total system, or else pressure fluctuations in the remaining portion of the system will result in unstable operation.

A special problem encountered in metering gases through flow restrictors operated by pressure gradients has to do with gases that have boiling points near ambient temperature. These gases, in their pure form, are obtained in pressure cylinders as liquids under their own vapor pressure. An example is nitrogen dioxide, which by all counts is one of the more difficult gases to handle. Its boiling point is 21.3°C, it is extremely corrosive, and it exists in both monomer and dimer form at ordinary temperatures. It can be handled through a rotometer if special precaution is taken. The principal features of such a system involve (1) provision of sufficient heat for the gas line and the rotometer to prevent condensation either through cooling from adiabatic expansion at the metering valve or through heat loss to the surroundings, and (2) use of materials made of stainless steel, glass, or Teflon. In the author's laboratory such a system has provided stable concentrations on a continuous basis over a period of months.

Evaporation Methods

If the concept of gas generation is extended to include those materials which are in the gaseous state at the conditions of exposure, the evaporation of liquids into the carrier gas stream can be included as method of generation.

Two general approaches to evaporation processes may be considered. The first is direct evaporation. Here a portion of the carrier gas stream is directed over or through the liquid of interest at such a rate that the stream becomes saturated with the vapor. This may be done with a bubbler or with a saturated

wick. Within limits the concentration of the active component in the tributary stream is

$$C = \frac{p}{P},$$

where

p = vapor pressure of liquid

and

P = total pressure,

and is independent of flow rate. Constancy of generation is dependent mainly upon maintaining isothermal conditions for the liquid reservoir.

Another type of evaporation process is that of diffusion from a liquid surface. A description of such an approach for low flows is given by Altshuller and Cohen.[12] In this process the liquid reservoir is in contact with the carrier gas through a capillary restriction, thus the rate of vapor addition will be proportional to vapor pressure, diffusion coefficient, and capillary dimensions, and will be independent of airflow. The concentration in the carrier gas, however, will be indirectly proportional to air flow.

Miscellaneous Chemical Methods

There exists a great variety of possible gas generation methods which can be classified roughly as chemical. More specifically they include the subcategories of electrolytic generation, chemical conversion, photochemical reaction, and high-voltage discharge production. These techniques have been used very little, with a few important exceptions. They are particularly useful when the material under consideration cannot be procured in a stable form, when it deteriorates, or when it creates a significant safety hazard. Hersch[5] has described several techniques for the chemical generation of gases, including one for the production of NO and/or NO_2 by electrolysis from a solution of nitrosyl hydrogen sulfate ($NOHSO_4$), and the production of ozone by ultraviolet radiation from a low-pressure mercury lamp.

An interesting possibility for the production of nitrogen dioxide from the more easily handled nitric oxide is oxidation by pure oxygen. This technique has been used in the analysis of mixed nitrogen oxides where the analysis method is for nitrogen dioxide. Little information is available as to large-scale use.

A commonly used method for producing large quantities of ozone is by the use of commercially available high-voltage-discharge ozone generators.

All of the above chemical methods of gas production, and most of the mechanical methods, require assays to determine actual concentrations. Even when fundamental principles can be used to calculate concentrations, losses of one type or another make dependence on them risky.

INTRODUCTION OF TEST GASES

The generation of test gases is but the first step in providing a controlled atmosphere within an exposure chamber. The method of gas transport, techniques and points of introduction to the carrier gas, the compatibility of mixtures, and safety are factors that must all be considered.

Gas Transport and Mixing

As a general principle the gas generation system should be as close as possible to the point of use to reduce the possibility of line losses. When safety considerations or lack of space dictate otherwise, close attention should be given to the use of nonreactive lines, gas-tight connections, and heat application if it is necessary to prevent condensation of high boiling gases.

In some cases predilution and transport can be combined. Rose and Brandt[2] have described the use of a two-stage, ram-air-driven venturi mixing system to move and dilute the raw exhaust of automobile engines. In this system a blower supplies carrier gas at a pressure sufficient to overcome venturi losses to each of the two stages which, in combination, could provide dilution ratios ranging from 600:1 to 3600:1.

The author has used a small stainless steel siphon, driven by a nonlubricated air pump, to predilute and transport nitrogen dioxide from a rotameter discharge to an exposure chamber. This technique has the advantage of reducing back-pressure on the nitrogen dioxide system and eliminating heating requirements after the siphon.

When predilution is not used, particular care must be given to ensure proper mixing before introduction to the test chamber. This may be done in most cases by introducing the test gas at the low-pressure side of the carrier gas system. Assuming that a centrifugal fan is used for the air-handling system, the turbulent action of the fan will produce good mixing. Reactive gases should be introduced into the air system after any chilling or heating elements have been used. Where conflicting requirements exist, the choice must be to reduce risk of reaction or loss and provide sufficient downstream turbulence to result in good mixing.

Carrier gas (breathing air) should be metered to permit calculation of predicted gas concentrations and to give indication of system performance. This is done best by measuring total flow with orifice or venturi meters. When velocity-measuring devices, such as pitot tubes and heated-wire anemometers (sometimes necessary to reduce pressure loss), are used, they should be located in long, straight duct sections, with flow straighteners incorporated if necessary. Velocity traverses across the cross section are necessary to establish the relationship of mean velocity with metering point velocity.

CONTAMINANT MONITORING

In any biologic study conducted to establish dose-response relationships some method must be used to establish the dosage of the agent being studied.

With complex agents the method of determining dosage is often such that it is difficult to make direct assay. In inhalation studies with cigarette smoke, for example, smoking machines have been devised[13–15] which attempt simulation of smoking by periodic introduction of short puffs of smoke to small animal chambers Assay is not done on this smoke, but rather on that produced in a similar fashion so as to provide sufficient material for characterization. The dosage is then defined indirectly by a statement of the exposure conditions and extraexperimental analysis.

In inhalation or other exposure studies, chambers larger than those designed to house a single small animal will generally have sufficient flow to permit direct analysis As discussed earlier, the inability to calculate accurately the contaminant concentration necessitates a confirming analysis Other important questions that may be answered by analysis concern contaminant distribution, contaminant loss, and the functioning of the dispensing and ventilation systems.

Of particular concern is the question of contaminant loss prior to or in the chamber. Vernot,[16] in his exposure chamber experience with toxic materials, found that twice the theoretical quantity of ozone, 1.5 times the nitrogen dioxide, and 1.2 times the carbon tetrachloride were required to reach desired concentrations. In a description of a large-scale animal study on the effects of diluted auto exhaust that had undergone various treatments, Hueter[17] stated that earlier chamber models gave 80–85% loss in ozone from supply to exhaust, 8–12% loss in nitric oxide, and 45–55% loss in nitrogen dioxide. New, larger chambers (44 ft^3) showed considerably less loss.

It is probable that much of the loss in small chambers is due to the presence of animals, since other chambers having specially fabricated nonreactive surfaces show far less loss Dimitriades[18] has described his experience with a 64 ft^3 chamber constructed of aluminum and Teflon and used in photochemical air pollution research In the dark, loss rates for O_3, NO, and NO_2 were O_3 – 12 hr half-life at 1 ppm, NO – negligible, NO_2 – 2% per hr at 1 ppm. The major problems he listed were associated with the flow and composition of reactant materials and with purification and flow of the air supply.

A very elaborate plant environmental chamber of approximately 500 ft^3 has been described by Hill.[19] It is constructed of stainless steel, with all the seams welded. Temperature, relative humidity, carbon dioxide, and air contaminant concentration are monitored with analyzers that provide feedback control to motor valves or other actuators

Sample Handling

Sample lines from chamber to analysis devices are potential sources of contaminant loss by adsorption or reaction. Thus, they should be constructed of noreactive materials, preferably glass, and no longer than necessary. One useful approach when the lines cannot be placed immediately adjacent to the chamber is to use all glass pipe, 1–1½ inches in diameter, with an auxiliary pump to provide an airflow substantially greater than required for analysis.

When test chambers are operated over a wide range of concentrations, one problem encountered in analysis with automatic continuous instrumentation is range capability. Most analyzers are optimized for fairly narrow concentration ranges. Rather than making internal adjustments it is often desirable to provide extended range capability by careful predilution. This problem is most pronounced with wet analyzers based on colorimetry. Only a few analyzers have a wide dynamic range; the flame ionization detector used for hydrocarbon determination is one that does.

Analysis Methods and Analyzers

The use of continuous monitoring instruments is at the present time limited to a relatively few of the gaseous air contaminants. The demand for such equipment, though rising, has not been great enough to stimulate manufacturers to produce instruments on a commercial basis, except for the more prevalent compounds.

The primary considerations in selecting such equipment include specificity, sensitivity, reliability, response time characteristics, ease of operation, and cost. The requirements for analyzers used with controlled test chambers may not be the same as those for analyzers primarily used in monitoring ambient air. For example, the West-Gaeke[20] colorimetric procedure for sulfur dioxide is inherently more specific than the conductometric method, but in an uncomplicated mixture of chamber test gas the simplicity and reliability of the conductometric method would strongly favor its use.

A relatively few measurement principles are used in commonly available continuous analyzers for gases. These principles, along with current applications, are given in Table 1. Continuous analyzers used for gases are far more prevalent

TABLE 1

Measurement Principles Used in Continuous Monitoring Instruments

Principle	Application
Absorption spectrometry	
Visible and ultraviolet	O_3, NO_2, SO_2, CHO^-
Infrared	CO, hydrocarbons
Chemiluminescence	O_3
Electrometric	
Conductometry	SO_2, acid gases
Coulometry	O_3, SO_2, and other electroreducible and oxidizable gases
Ionization (flame)	Organics
Electron capture*	Peroxyacylnitrates, halogenated organics

*Semiautomatic sequential analysis with gas chromatography.

for inorganic compounds than for organic ones. This is in part a result of the generally more complex structure of the organic compounds, which makes the analysis problem more difficult.

Two brief examples can be used to illustrate the salient features of analysis techniques adapted for continuous analyzers in general use. First is the use of near ultraviolet and visible absorption spectrophotometry. This technique is commonly used where a colored reaction product of the compound under consideration and a specific reagent can be developed in a continuous contactor and the product measured in an optical-flow cell.

With this class of analyzer, it is necessary to meter both the sample and reagent flow, to provide an appropriate contactor, to separate the developed reagent from the sample airstream, to measure the light absorption in a flowing optical cell, and to record continuously the signal output from the detector. Analyzers of this type are distinguished by their generally high sensitivity, and by the close attention necessary in calibration and operation.

Substances currently measured by this technique include ozone (release of iodine from potassium iodide), nitrogen dioxide (formation of an azo dye with Saltzman's reagent), and sulfur dioxide (formation of pararosaniline methysulfonic acid with the West-Gaeke reagent).

A quite different monitoring technique can be used if the compound to be measured has some physical property susceptible of being quantified. Thus, by use of the Luft technique, carbon monoxide can be measured by the use of infrared photometry without the necessity of a reagent. A typical analyzer of this type consists of an infrared source, sample and reference gas cells, detector cell, amplifier, recorder, and the necessary controls. An infrared radiation is passed through the sample cell and the reference cell (containing a nonabsorbing gas such as N_2), the result being that a varying amount of infrared energy, indirectly proportional to the carbon monoxide concentration in the sample cell, reaches the detector. The detector selectively responds to the infrared energy absorbed by carbon monoxide, by virtue of being filled with that gas and ultimately produces a signal which is amplified and fed to a recorder.

A direct physical measurement such as described above has several advantages, the chief one being the absence of a reagent and the relative insensitivity to sample flow variations. The disadvantages are principally related to the relatively low signal-to-noise ratio and the more elaborate provisions for signal conditioning and amplification.

Data Evaluation

The productive use of continuous analyzers requires close attention to calibration, operation, and maintenance. Data are usually produced on time-based continuous strip charts, although tape recorders and analog-to-digital converters are increasingly being used in systems with a high volume of data. In operations on a smaller scale, data are usually reduced by hand after an editing

process. Particular care is necessary in handling output from colorimetric-type analyzers because of the logarithmic relationship between signal and concentration

REFERENCES

1 J P Lodge, Production of controlled test atmospheres In, *Air Pollution*, 2nd ed , Vol II, pp 465–483, A C Stern (ed) Academic Press, Inc , New York, N Y , 1968
2 A H Rose, Jr , and C S Brandt, Environmental irradiation test facility *J Air Pollut Control Assoc* , 10(1960) 331–335
3 R J Bryan and W M Blackmore, Controlled atmosphere exposure chamber *Arch Environ Health,* 18(1969) 848–854
4 S D Silver, Constant flow gassing chambers Principles influencing design and operation *J Lab Clin Med* , 31(1946) 1153
5 P A Hersch, Controlled addition of experimental pollutants to air *J Air Pollut Control Assoc* , 19(1969) 164–172
6 P A Hersch and E J Whittle, Injecting trace impurities into a gas stream *J Sci Instr* , 35(1958) 32–33
7 G O Nelson and K E Griggs, Precision dynamic method for producing known concentrations of gas and solvent vapor in air *Rev Sci Instr* , 39(1968) 927–928
8 M D Thomas and R E Amtower, Gas dilution apparatus for preparing reproducible dynamic gas mixtures in any desired concentration and complexity *J Air Pollut Control Assoc* 16(1966) 618–623
9 W Holl and R Muhleisen, *Geofis Pura Appl* , 31(1955) 21
10 B E Saltzman and A F Wartburg, Precision flow dilution system for standard low concentrations of nitrogen dioxide *Anal Chem.*, 37(1965) 1261
11 J D MacEwen, in *Proceedings of the Conference on Atmospheric Contamination in Confined Spaces*, AMRL-TR-65-20, Aerospace Medical Laboratories, 1965.
12 A P Altshuller and I R Cohen, Application of diffusion cells to the production of known concentrations of gaseous hydrocarbons *Anal Chem* , 32(1960) 802
13 R H Holland, R H Wilson, D Morris, M S McCall, and H Lanz, The effect of cigarette smoke on the respiratory system of the rabbit *Cancer*, 11(1958) 709–712
14 G E Moore and F G Bock, A summary of research techniques for investigating the cigarette smoking-lung cancer problem *Surgery*, 39(1956) 120–130
15 D Hoffman and E L Wynder, *Tobacco and Tobacco Smoke* Academic Press, Inc , New York, N Y , 1967
16 E H Vernot, in *Proceedings of the Conference on Atmospheric Contamination in Confined Spaces*, AMRL TR-65-20, Aerospace Medical Laboratories, 1965
17 F G Hueter, in *Proceedings of the Conference on Atmospheric Contamination in Confined Spaces*, AMRL-TR-65-20, Aerospace Medical Laboratories, 1965
18 B Dimitriades, Methodology in air pollution studies using irradiation chambers *J Air Pollut Control Assoc* , 17(1967) 460–466
19 A C Hill, A special purpose plant environmental chamber for air pollution studies *J Air Pollut Control Assoc* , 17(1967) 743–748
20 P W West and G C Gaike, Fixation of sulphur dioxide as disulfitomercurate and subsequent colorimetric estimation *Anal Chem.*, 28(1956) 1816
21 M D Thomas, J O Ivie, and T C Fitt, Automatic apparatus for the determination of small concentrations of sulphur dioxide in air *Ind Eng Chem. Intern Ed* , 18(1946) 383

DISCUSSION

Y. Alarie: I am wondering if you can give us some approximate costs to show people what it really takes to finance some inhalation studies With those large chambers, and using only 0 1 of 1% of the gas that you dispense, they can be quite expensive, particularly with chronic studies

R. J. Bryan. Well, I think it depends on your point of view Incidentally, these chambers never had more than about a half a percent of their volume occupied, and I've yet to see in any of these systems, as opposed to an unoccupied chamber, any indication whatsoever of absorption of even the most reactive gases That happens to be one advantage

It's true that to operate at around 20 ppm of NO_2 in that system we have to use about two 10-pound cylinders of NO_2 a week, and this runs about $50 or $60 a week On the other hand, if you look at personnel costs and other costs, such as overhead, I think in general you will find that in an overall budget, materials are a rather insignificant cost of research That's a personal opinion I don't mean to say that it isn't important It may make the difference, sometimes, whether you get to do something or not, but my personal opinion is that I don't think it's very significant, even though it is a dollar amount, to talk about $50 or $60 a week.

SESSION II

CELLULAR AND FUNCTIONAL INJURY FOLLOWING INHALATION EXPOSURE

Chairman – Clayton Loosli
University of Southern California
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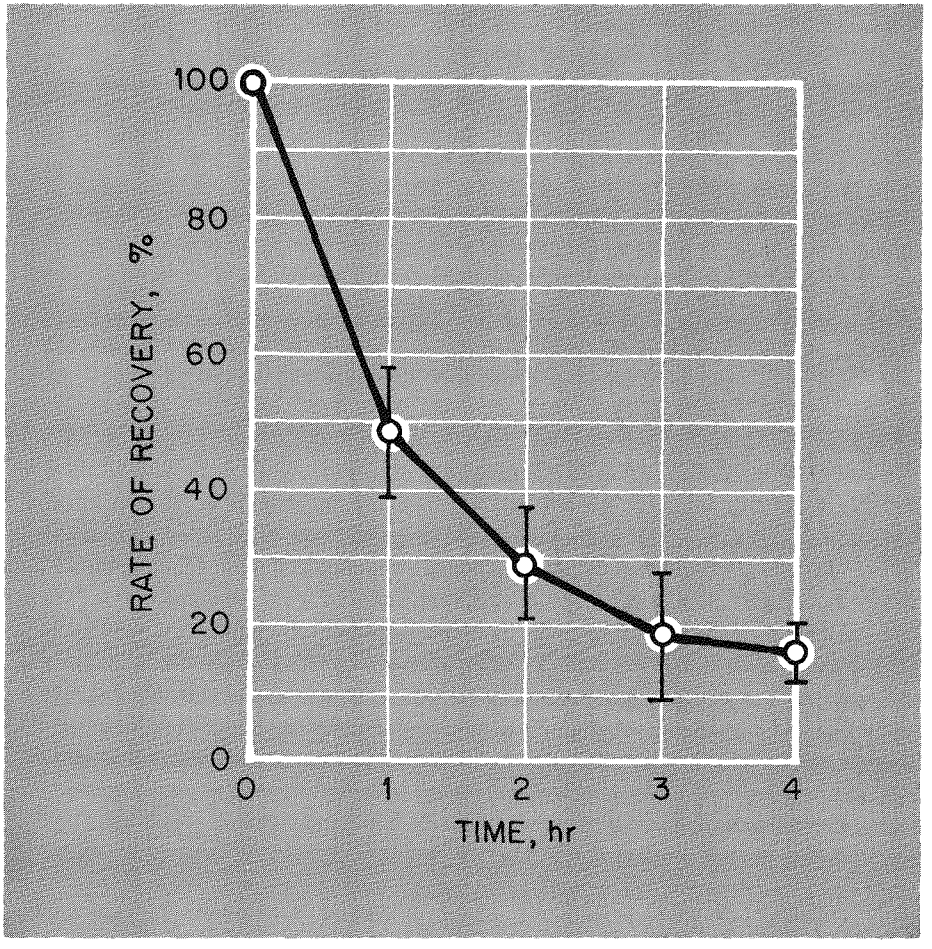

BIOCHEMICAL PARAMETERS IN INHALATION CARCINOGENESIS

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ABSTRACT

Studies are reported which indicate that two insoluble lung proteins, collagen and elastin, are altered (denatured) following exposure to either ozone or NO_2 Electron micrographs of collagen fibers isolated from the lungs of exposed rabbits appear to confirm this It may be significant that denaturation has been ascribed a role in carcinogenesis — Evidence of lipid peroxidation *in vivo* after exposure to either ozone or NO_2, and the possible consequences of peroxidation in cells and cellular membranes, are discussed Since the presence of peroxides implies the presence of free radicals, some of the evidence linking the latter to cancer is summarized — Spectral characteristics of DNA isolated from rabbit lung and the interaction of DNA with polycyclic hydrocarbons is discussed, as is a possible role for air pollutants in carcinogenesis — Known biochemical parameters of nonhydrocarbon, airborne carcinogens are reviewed, and potentially productive areas of research are outlined

Air pollution, or photochemical smog, has been implicated in bronchitis, emphysema, and lung cancer At the present time, conclusions about the role of atmospheric pollution in cancer are based on epidemiological findings For example, a recently completed study, covering a 9-year period and 70,000 Californians, concluded that smog is not a causative factor in lung cancer [1,2]

A wider view may be that of Clayson,[3] who finds that epidemiological surveys of large populations indicate that many cancers have an environmental origin The nature of the agents involved is a matter for speculation that must be guided by the experimental study of chemical carcinogenesis Even now, to paraphrase Clayson, potent new synthetic carcinogens, which do not belong to the well-established classes of chemical carcinogens, are still being found, some of which may induce cancer after only a single dose

The work in this laboratory has been aimed at studying the effects of ozone and NO_2, two potent components of photochemical smog, on several lung

components, including collagen, elastin, lipids, and DNA. I shall attempt to relate these and others to inhalation carcinogenesis.

PROTEINS

The isolation and purification of insoluble collagen, one of several collagen forms, has been described previously.[4] Though normally inert and insoluble, we have been able to solubilize both this collagen and elastin, and to study their structure by modifying techniques of differential ultraviolet spectrophotometry.[5,6] This has enabled us to demonstrate that both proteins are altered in structure (denatured) after exposure to either ozone or NO_2. Since denaturation is shown by a shift in the protein peak to a shorter wavelength, we feel justified in referring to the altered structure as denaturation, or change in conformation.

Figure 1 is an electron micrograph of collagen isolated from the lungs of rabbits exposed to 5 ppm of ozone for 1 hr. The change in conformation, or folding specificity of the protein, is evident. The normal collagen is undoubtedly derived from the lung vasculature, of which collagen is an important component.

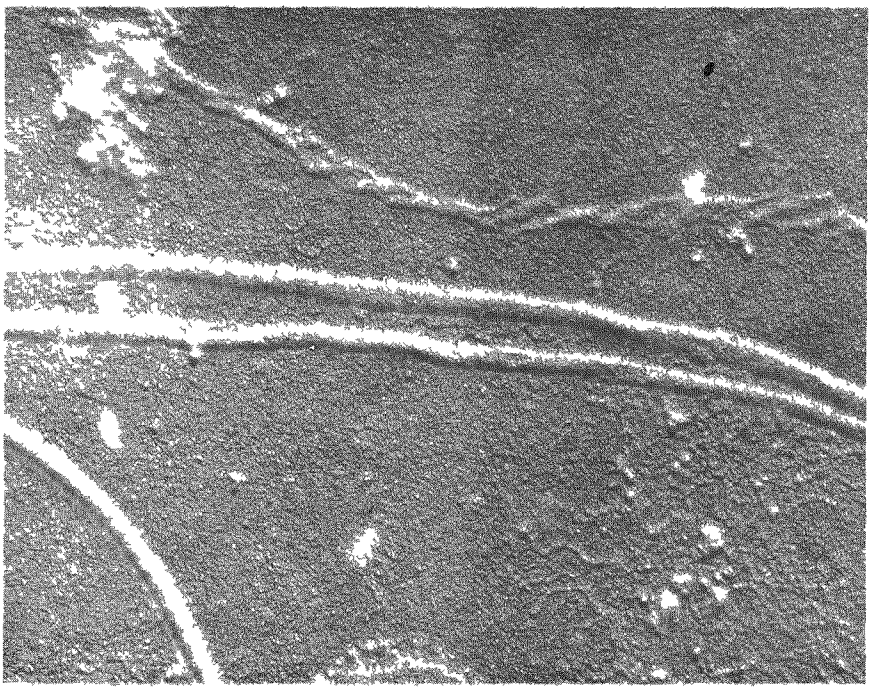

Fig. 1 – Insoluble collagen isolated from lung of rabbit exposed to 5 ppm of ozone for 1 hr. Denatured collagen, left; normal collagen probably derived from lung vasculature, right. Shadow cast with uranium oxide. (73,000X)

Although some types of denaturation are reported to be reversible, it is difficult to reproduce the folding specificity of a protein to its native state, once it has been denatured.[7] It appears significant that tobacco smoke condensate also alters the structure of collagen and elastin.[8]

Denaturation has been linked with cancer or with carcinogenesis. For example, Moriyama and Ohashi[9] considered viruses as denaturing agents that induce in cells the formation of new virus radicals that are then released, enter other cells, and repeat the process. Although presented some years ago, this thought is not inconsistent with present thoughts about virus reproduction.

Rondoni[10] regards physical and chemical carcinogenic agents as denaturing factors, producing in those protein systems related with inner cell organization, and with differentiation, a particular type of denaturation or disorganization that does not inhibit or interfere with normal cellular functions, such as cellular multiplication. He further points out that protein denaturation and the carcinogenic mechanism have many physical properties in common.

O. Schmidt (cited in Rondoni[11]) reported that carcinogenic agents react with some protein system to induce the formation of a more stable denaturation product; the energy released would be utilized in the uncontrolled cellular proliferation so characteristic of cancer.

Alteration in cell surface components in carcinogenesis is emphasized by Rubin,[12] who views the malignant transformation as arising from heritable disruption in the distribution and configuration of cell surface macromolecules. Once a cell surface is sufficiently altered, Rubin goes on, it can no longer be restored to normal, and the cell becomes progressively more malignant as the pattern approaches a random distribution of molecules. This describes the phenomenon of denaturation very well.

Although seemingly unrelated, it may be significant that Pace[13] has reported that 4 ppm of ozone alters the collagen-like substance through which cells attach themselves. This substance may be associated with the phenomenon of "contact inhibition" exhibited by cancerous cells, which appears to be the result of greater adhesion of a cell to its substrate than to another cell.[14] Cells escape from contact inhibition only after they become malignant, and then are capable of invading other tissues, or metastasizing, suggesting extensive changes in the cell membrane.

This is substantiated by time-lapse photography with the interference microscope. Films show that a ruffled membrane forms on the leading edges of normal cells, whereas sarcoma cells become rounded and tend to produce a number of independent pseudopodia along with the ruffled membranes, an actual physical change or deformation.[15] A few hours after skin has been painted with a carcinogen, the mutual adhesiveness of epithelial cells decreases.[16]

Scanning electron microscopy has recently revealed differences in the surfaces and intracellular contacts between normal and polyoma transformed (malignant) tissue culture cells *in vivo*.[17] Although we are aware of the

importance of the cell membrane and its components, we have been unsuccessful in isolating the protein component

COLLAGEN

We have observed physical damage to insoluble collagen fibers isolated from the lungs of rabbits exposed to NO_2 for relatively long periods, as shown in electron micrographs Four litter-mate rabbits were exposed to 0 25 ppm NO_2, three of the four litter-mates were exposed simultaneously 4 hr daily, 5 days a week, to 0 25 ppm of NO_2 and killed at intervals One was killed after 24 days of exposure, and one of the two remaining rabbits, after 36 days The last rabbit, also exposed for 36 days, was returned to the animal colony and killed 7 days later The control rabbit was exposed to air under ambient conditions in a duplicate of the exposure chamber for 4 hr a day for 12 days and then killed Electron micrographs of the isolated collagen were prepared and sent "blind" to a laboratory consultant in electron microscopy for evaluation His comments are summarized below [18]

1 Control (Fig 2) Fibrils appeared normal except in the places noted by arrows

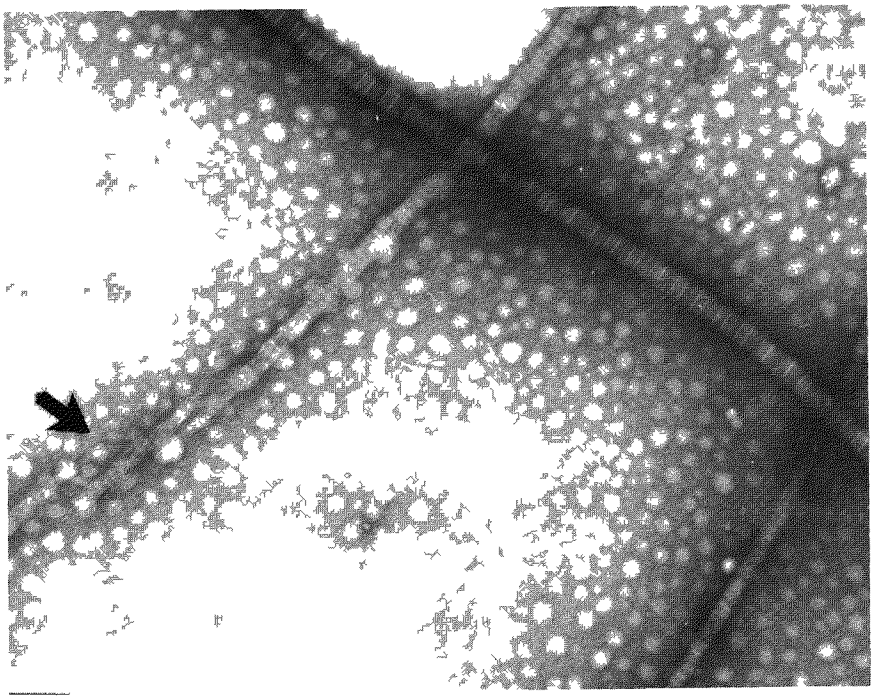

Fig 2 Insoluble collagen isolated from lung of rabbit exposed 4 hr a day, for 12 days, to air under ambient conditions (20,000X)

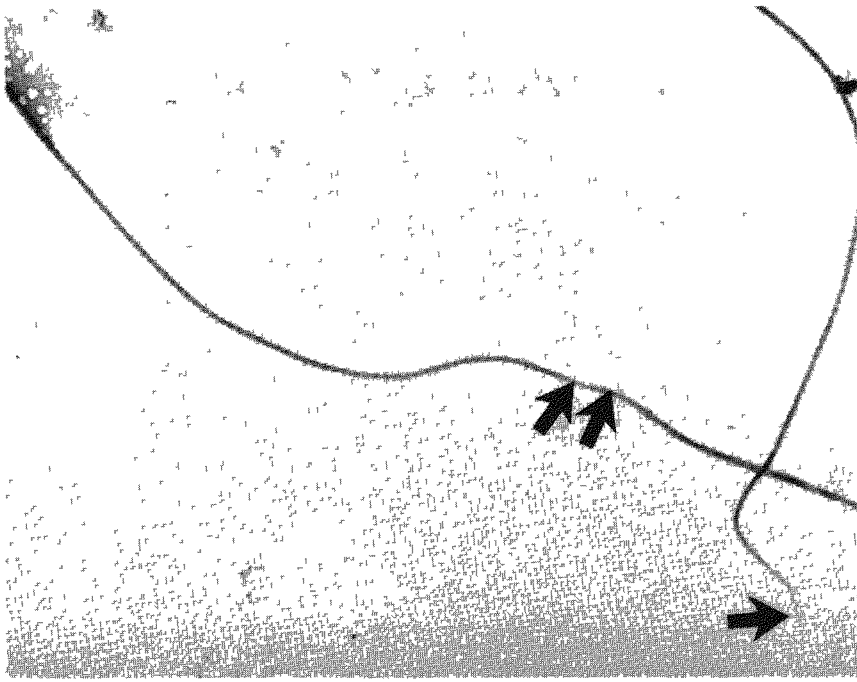

Fig. 3 – Insoluble collagen isolated from lung of rabbit exposed to 0.25 ppm of NO_2 4 hr a day, for 24 days. (20,000X)

2. Exposed 24 days (Fig. 3): The collagen in these micrographs may not have been normal. The non-normal feature was the finite length of the fibrils and the twisting and tapering of the fibril ends.

3. Exposed 36 days (Fig. 4): Partially degraded, twisting, swelling, and tapered fibril ends, and finite length.

4. Exposed 36 days, killed 7 days later (Figs. 5 and 6): These samples appear normal.

If one assumes the formation of scar tissue as a result of such lesions, there may have been a potential for cancer in these animals. Rossle[19] has described scar carcinomas arising in old pulmonary scars containing deposits of cholesterol. He regarded the combination of scar and cholesterol as chemically carcinogenic. Gillman *et al.*[20] report that collagen in scars, if subjected to repeated tears, suffers degeneration and may become the stroma of developing cancer. They also report that squamous cell and adenocarcinoma of human lung, which commonly arise in the periphery, are usually associated with scars. Raeburn and Spencer[21] also implicate scars in carcinogenesis.

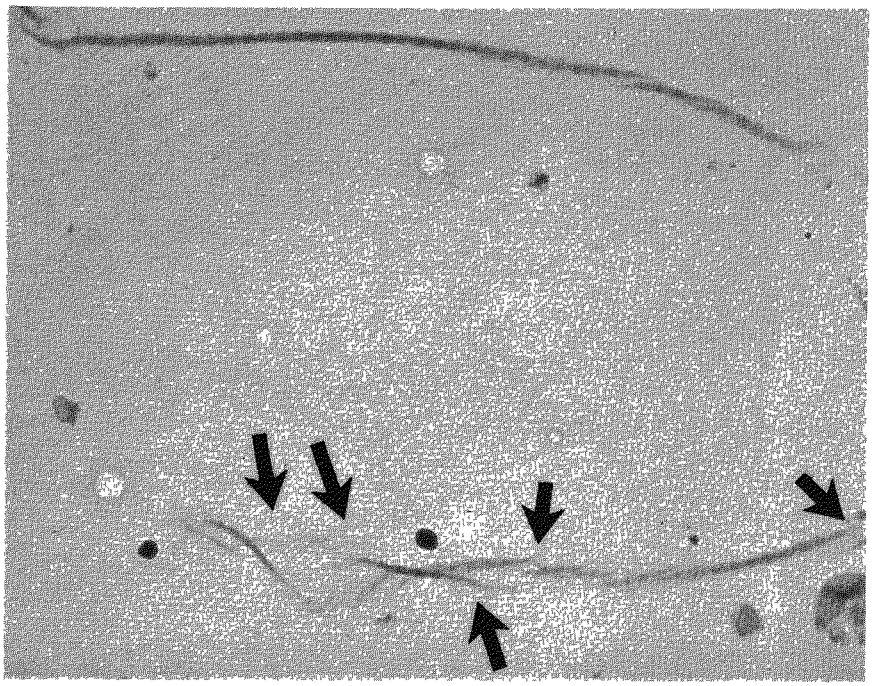

Fig. 4 – Insoluble collagen isolated from lung of rabbit exposed to 0.25 ppm of NO_2 4 hr a day, for 36 days. (12,000X)

Figs. 5 and 6 – Insoluble collagen isolated from lung of rabbit exposed to 0.25 ppm of NO_2 4 hr a day, for 36 days, and killed 7 days later. (12,000X)

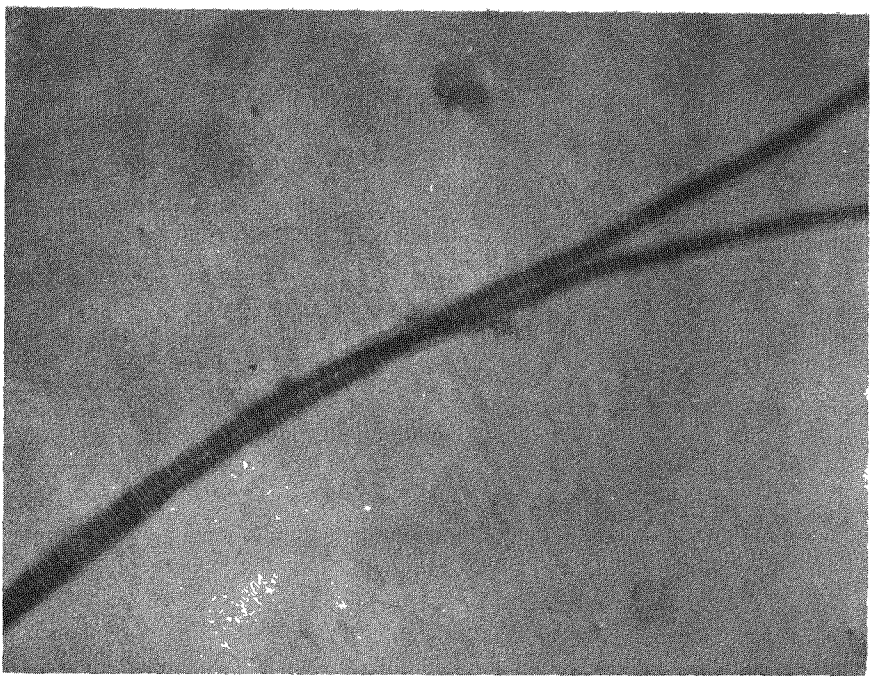

Figure 5

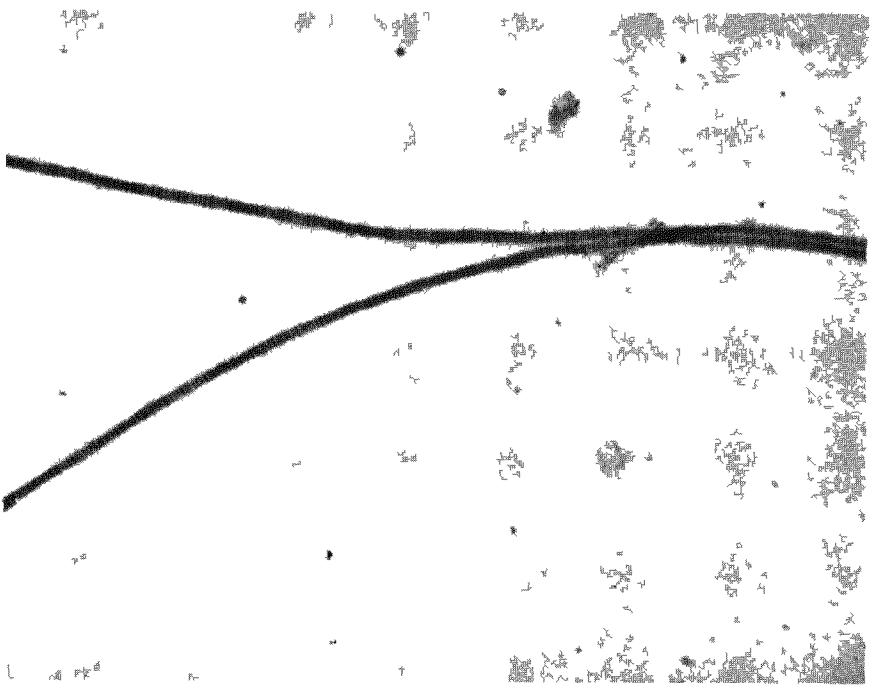

Figure 6

IMMUNOLOGY

Since a feature of the malignant cell is a disturbed control of protein synthesis,[22] an abnormal protein system that synthesizes proteins with a new intramolecular configuration (denaturation) could involve immunologic specificity. In fact, Peters and Goetzl[23] were able to show that thermodynamically stable, but conformationally altered, reoxidized monomer albumin possessed greater antigenic reactivity than native monomer albumin. Although chemical or physical oncogenic stimuli result in the appearance of tumor-specific antigens in the target tissue, the reason for this unique antigenicity is unknown.[24]

There are a number of well-substantiated reports of tumor-specific antigens. Zilber[25] and his group, for example, have described antigens present in human carcinomas of the liver, esophagus, mammary gland, lung, and other tissues.

Tumor-specific immunity is only a special case of transplantation immunity, and much of the evidence that has established the presence of new, foreign antigens has been obtained through techniques involving tumor-specific transplantation antigens, demonstrated by immunizing animals against a transplant challenge.[26] They have been detected in most malignant tumors, including carcinogen-induced skin carcinomas in mice. They probably result from new information resulting from a change in DNA, or as a result of derepression of existing information.

Lappé,[27] however, has recently presented evidence that a major portion of the antigenicity present in premalignant lesions can persist unchanged through progression to malignancy. What are detected as tumor-specific transplantation antigens in other malignant tumors may be derivatives of antigens present in earlier tumor pathogenesis, as predicted by Burnet's theory of immunological surveillance. This theory envisions the microscopic elimination of antigenic tumors as the result of an active immunologic process. The idea that cancer can be facilitated by impairment of immunologic processes is supported by the observations of Barden, Good, and Smith. Barden[28] finds that collagen diseases, such as scleroderma and lupus erythematosus, and cancer have a common factor – a disturbance in globulin synthesis. Therefore, Barden suggests that immunoproliferative and immunodeficient states may provide a bridge between collagen diseases and cancer. Good[29] also considers immunological deficiency an important element in the development of neoplasms, while Smith[30] finds that evidence of immunologic deficiency always antedates appearance of tumors.

In general, the neoplastic transformation of cells, tumor formation, involves the acquisition of new and tumor-specific antigenicity.

If there are antigens, there must be antibodies. Early work indicated that antibodies were formed against pulmonary tumors, but not against normal lung.[31] Later, antigens were prepared by adsorbing proteins of human tumors on aluminum cream. Six months after injection of the antigen into rabbits, they had developed antibodies against the neoplastic proteins but not against others that were tested. Specificity was evident even at high dilutions.[32–34]

Gorer[35] presented a clear-cut example of immunologic rejection by antibodies, by showing that serum from mice which had rejected a tumor would, when mixed *in vitro* with live leukemia cells, prevent their subsequent growth.

More recently, De Carvalho,[36,37] after extracting protein antigens from human normal and cancer tissue with fluorocarbon solvents, adsorbed the antibodies against normal tissue on human normal antigens, thereby isolating cancer antibodies, which were then administered to terminal patients.

Immunotherapy of malignant disease involving cross-transplantation and leukophoresis shows promise as a therapeutic measure, causing regression and even disappearance of tumors.[38] Further emphasizing the immunological component in carcinogenesis and cancer, it has been found that malignancy can be suppressed when malignant cells are fused with nonmalignant cells, suggesting a new form of immunotherapy.[39]

LIPIDS

We have also examined the lipid fraction of rabbit lung tissue, the isolation of which has been described previously.[40] One could expect any unsaturated lipids present to be cleaved at the double bond to give carbonyls (aldehydes or ketones), an hypothesis verified by experiment. Further experiments to confirm the formation of carbonyls following ozone exposure will be reported later.

Perhaps the simplest mechanism to account for their formation is the following simplified reaction, utilizing the Criegee mechanism:

$$RCH = C\,R'R \xrightarrow{O_3} RCH(O_3)\,C\,R'R'' \longrightarrow RCHO + R'COR''$$

Only those carbonyls above C_6 were considered as derived from the various lung lipids, to exclude those derived from lung proteins.

Some of the aldehydes and semialdehydes which may be present, and their fatty acid source, are:[41]

Fatty acid	Aldehyde	Semialdehyde
Linoleic	Caproicaldehyde	Azelaic
Linolenic	Propionaldehyde	Azelaic
Arachidonic	Caproicaldehyde	Adipic
Tetradecenoic	Valeraldehyde	Azelaic
Hexadecenoic	Heptaldehyde	Azelaic

In addition to neutral lipids in the cellular membranes, another source of the lipid-derived carbonyls is probably the 25% lipid, mainly highly unsaturated fatty acids, found in the mitochondria within the cells. A third source might well be the plasmalogens, which appear to be ubiquitous.

At least a portion of the aldehyde component of the carbonyls, formed by the interaction of lipids and ozone, may have a secondary effect, since there is

evidence that some aldehydes may have the potential of reacting with DNA and change its properties, even to uncoiling the two-stranded DNA molecule.[42]

Both ozone and NO_2 cause increased formation of diene bonds in lipids *in vivo,* which is characteristic of lipid peroxidation.[43,44] The latter are highly stable, even to catalase,[45] and give rise to free radicals, which are able to migrate through biological membranes.[46] If they reach sensitive sites in the cell, they may interfere with normal cell functions.[47]

Other studies have shown that biological membranes, largely phospholipid and protein in nature, are extremely labile to lipid peroxidation.[48] A disrupted cell surface, such as that which must occur after exposure to ozone or NO_2, could conceivably favor the deposition of carcinogenic hydrocarbons, which appears to be essential for hydrocarbon-induced carcinogenesis.[49]

It may be relevant that free radical activity and the concentration of paramagnetic ions in some cancerous tissue differs markedly from those in normal tissue, with the overall intensity of electron paramagnetic resonance signals several times larger in cancer tissue than in normal tissue.[50] This suggests an increased concentration of paramagnetic ions in the cancerous tissue, probably derived from a metabolic disturbance caused by the cancer. Similar findings have been reported by Emanuel *et al.*[51] and by Nebert and Mason (cited in Morris[52]).

Although polluted air contains a relatively high concentration of free radicals, as does air containing ozone and smoke derived from the burning of organic material,[53] the significance, if any, of these airborne free radicals in health or carcinogenesis is unknown.

DNA-POLYCYCLIC AROMATIC HYDROCARBONS

Apparently, the interaction of polycyclic aromatic hydrocarbons with DNA is the principal or primary event in hydrocarbon-induced cancer, so the two should be considered together. DNA is located within the nucleus, but it is also associated with mitochondria[54] and even microsomes.[55] Since conversion of normal cells to neoplastic cells is heritable, it is evident that DNA must play a vital part in the transformation.

Our interest in DNA was stimulated by the report that ozone is mutagenic in bacteria, indicating some change in DNA.[56] This does not mean that ozone is carcinogenic.

A number of methods for the isolation of DNA were studied and compared. We have found the most satisfactory method to be that of Orlov and Orlova,[57] with a slight modification. Full details will be published later. Identity of the isolated DNA has been verified by infrared spectroscopy, by absorption at 260 $m\mu$, and by some arbitrary wavelength ratios used in establishing the purity of DNA. Typical data are shown in Table 1. Evidently, DNA isolated from rabbit lung tissue compares favorably with commercial DNA.

TABLE 1

Spectral Characteristics of DNA Isolated from Rabbit Lung and Other Sources

	260/280 Ratio	260/230 Ratio
Animal		
0	1.96	1.62
1	1.89	1.58
5	1.92	1.52
Duplicate of above		
0	1.96	1.61
1	1.95	1.60
5	1.91	1.60
Commercial DNA	1.81	1.58
Sperm DNA	1.94	1.54

In polluted atmospheres of U.S.A. cities, suspended particles contain approximately 0.5% large hydrocarbons and only about 0.03% total polynuclear aromatic hydrocarbons.[58] Because of their steroidal configuration and solubility properties, the aromatic hydrocarbons are believed to localize in the cellular membranes, again emphasizing the significance of cellular membranes.[59]

There have been many attempts to correlate the molecular and/or electronic structure of the aromatic hydrocarbons and their carcinogenic potential. Pataki and Huggins,[60] for example, have found that positions 6, 7, 8, or 12 in derivatives of benz[*a*]anthracene form a triangle of carcinogenicity when they possess two or three methyl groups in any combination (Fig. 7).

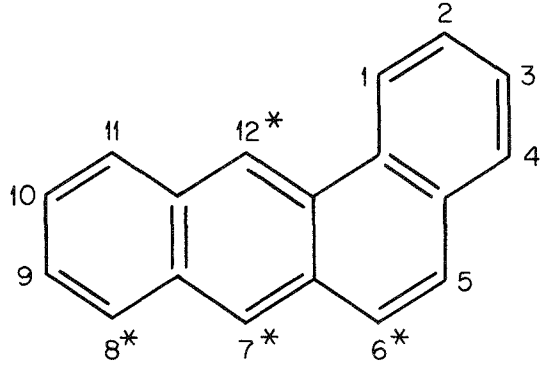

Fig. 7 – Benz[*a*]anthracene.

Hydrocarbon and nucleic acid interaction has been detected by equilibrium dialysis, by the determination of enhancement in viscosity in dilute solutions, and by measurement of absorption and excitation spectra. So, there is little doubt they interact. There is considerable evidence that polynuclear hydrocarbons interact with DNA as such, and do not require metabolic activity for carcinogenicity. A number of carcinogens bind covalently to both proteins and nucleic acids, but hydrophobic bonding is probably the sole interaction between polycyclic hydrocarbons and protein-lipoprotein during transport.[61]

On the other hand, Brookes and Lawley studied the binding of carcinogens to cellular constituents in mouse skin carcinogenicity. They report a strong correlation between carcinogenicity of the hydrocarbons used and their binding to DNA, with maximum binding occurring after 24 hr, which suggests that metabolism of the hydrocarbons was essential for reaction with cellular constituents.[62]

Among specific DNA and RNA components that show a significant interaction with aromatic hydrocarbons are adenosine, uridine, thymidine, and guanosine. Although the potent carcinogenic compounds are all bound moderately well, the strength of binding is related more to the number of aromatic rings than to the expected carcinogenicity of the hydrocarbons.[61]

At least one carcinogen, acetylaminofluorene,[63] interferes with normal protein synthesis, apparently by modifying transfer RNA (tRNA). This permits the study of how carcinogens act at the translation level at which each amino acid is bound by a specific tRNA molecule and transferred to the protein assembly line. This may be a common action of all carcinogens.

Pullman postulates[64] that the initial interaction between polynuclear aromatic hydrocarbons and possible cellular receptors (proteins or nucleic acids) is a physical attraction followed by chemical (enzymic) bond formation. Independent experiments by Daudel and Terayama (cited in Weisburger *et al.*[64]) indicate that carcinogens lead to a deficiency of ribosome messenger units. The constantly changing concepts on mechanism of activation of chemical carcinogens and their interaction with cellular and molecular receptors are summarized in a recent publication.[64] It is apparent that the biochemistry of carcinogen-tissue interaction is not clear at this time. Di Paolo *et al.*[65] have described an *in vitro* model for studying chemical-induced oncogenesis, which hopefully may prove useful in elucidating the mechanism of this type of carcinogenesis.

Since HNO_2 is believed to be formed when the air pollutant NO_2 and water react, it is a possibility that such a reaction might occur *in vivo* in the lungs, assuming that HNO_2 is stable, and that it eventually reaches the nucleus. Another remote possibility is that deamination of guanine, adenine, and cytosine might occur, and even result in cross-linking of DNA, thus preventing replication.[66]

Laskin and Kushner[67] recently reported that experimental animals pre-exposed to SO_2, another air pollutant, and then exposed to inhaled polynuclear hydrocarbons, developed a true metastasizing bronchial carcinoma. This sug-

gests, and suggests only, that the common air pollutants, including cigarette smoke, may act as an initiator,[68] which has been described as a type of incomplete carcinogen that brings about the first stage in the two-stage mechanism of carcinogenesis. As the next logical progression, air pollutants might also be regarded as promoting agents,[68] which, through repeated irritation of the bronchial mucosa,[69] might facilitate appearance of the second stage in the two-stage mechanism of the carcinogenesis. This is analogous to film carcinogenesis, in which Danishefsky and co-workers find that some of the stimulated cells (irritation) may eventually respond by abnormal, neoplastic growth.[70]

OTHER AIRBORNE CARCINOGENS

In addition to polynuclear aromatic hydrocarbons, other airborne carcinogens are known. Statistical studies correlating pulmonary, pleural, and peritoneal carcinomas and mesotheliomas of humans with asbestosis or asbestos dust exposure, as well as experimental studies on animals exposed to asbestos, conclusively implicate asbestos as a carcinogenic agent.[71]

Asbestos has been described as an irritative carcinogen, and this so-called irritative effect, as well as fibrosis, is characteristic of solid-state carcinogenesis.[71]

Gross *et al.*,[72] in their study of lung cancer in rats exposed to chrysotile asbestos, hypothesize that cancer production involves trace metals introduced during hammer milling of the chrysotile. They found inhaled asbestos dust early becomes coated with an iron-containing protein to form asbestos bodies, which can be recognized within 7 days after exposure to asbestos dust. Holt *et al.*[73] found that inhaled asbestos dust quickly becomes coated with an iron-containing protein to form asbestos bodies.

Although a causal relationship between silicosis and cancer was not universally accepted by early investigators, a survey based on recent animal experimentation and statistical studies on humans indicates that such a relationship can exist.[74]

It is believed that silicic acid is the toxic substance that diffuses out of the silica and stimulates the formation of collagen.[75]

Guinea pigs exposed to a silica dust cloud of about 40,000 particles/ml (0.3–3.0 μ) and killed at intervals showed an increase of lung lipid and phospholipid paralleling a rise in collagen.[76] Collagen and ascorbic acid levels appear to be involved with the onset of silicosis, ascorbic acid deficiency resulting in retarded collagen fiber formation.[77,78]

Arsenic, long suspected as a carcinogen, has been firmly established as a carcinogen by Lee and Fraumeni.[79]

Pure chromium compounds and the dust of roasted chromite all produce pulmonary tumors in rats and mice in the absence of any carcinogenic hydrocarbons. In this country, the incidence of lung cancer in some chromate

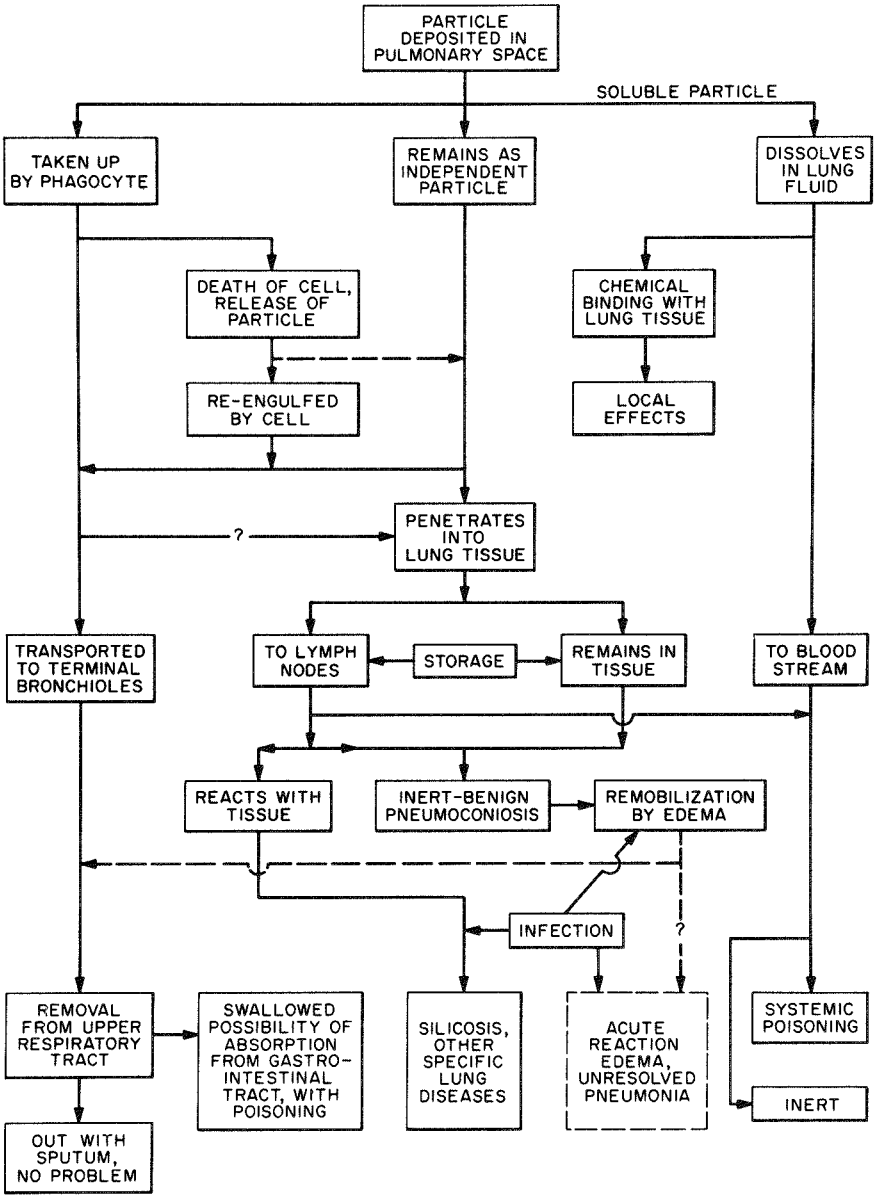

Fig 8 – Fate of inhaled particles (Reproduced by permission of author and publisher[91])

works appear to be about 3.5 times that found in the general population. The scanty evidence available indicates that proteins and nucleic acids are altered by chromium. The idea that chromium might act as a promoting agent for other carcinogens such as 3,4-benzopyrene has been examined, but the latter substance causes more tumors alone than when chromium is added.[80]

The role of inhaled nickel in carcinogenesis is uncertain, although in a single experiment rats developed cancer 2 years after a massive dose of nickel carbonyl.[81] Hueper[82] has found that inhaled nickel produced sarcoma in rats and rabbits, and epithelial tumors in guinea pigs, so it must be suspect.

Beryllium appears to be carcinogenic in the rat and some other animals, but not so far in human beings.[83] Vorwald[84] found primary bronchogenic carcinoma in animals exposed to continuous inhalation of low concentrations of beryllium oxide and sulfate. Available evidence suggests that beryllium appears to affect the distribution of lipid, RNA, and DNA. It inhibits some enzymes, but activates others.[85] Berylliosis also appears to have a poorly defined immunological component.[86,87]

Lead and cadmium have also been implicated as carcinogens, though not necessarily through inhalation.[88]

Manganese affects the whole respiratory system, but so far no carcinogenic effects have been shown.[89]

As with the polynuclear aromatic hydrocarbons, the common cellular constituents involved are proteins, lipids, and nucleic acids.

It should not be surprising that the biochemical changes following inhalation of these varied carcinogens are essentially the same as those observed when plastics or metals are imbedded in rodents.[90] Research in this area is practically nonexistent, presumably because preventive measures are being practiced to some extent. Figure 8 shows the fate of inhaled particles.

Although there are probably many biochemical differences between normal and tumor tissue, most may not have any relevance to the unusual characteristics common to most, if not all, cancers: metastasis, cellular multiplication, and loss of normal control mechanisms.[92] However, there appears to be a genuine trend in several laboratories toward more meaningful comparisons between normal and tumor tissues.[93] The key word here is, obviously, *meaningful.*

SUMMARY

It is evident that advances in cancer biochemistry must parallel developments in general biochemistry, which, as this brief discussion has attempted to point out, offers several promising areas of research, such as:

1. Immunology. To quote Haughton and Amos,[94] "the increasing number of well-substantiated reports of tumor-specific antigens suggest that an intensive experimental attack using modern immunologic and genetic techniques is somewhat overdue."

2. Cell fusion, which is closely allied to immunology and offers a chemical method for studying the mechanisms by which malignancy can be suppressed.
3. Mechanism(s) of carcinogenesis, approached by the method of Di Paolo *et al.*[65]
4. Studies on the mechanism of gene expression or gene activity, and the study of repressors and how they function.[95] Recent reports indicate that progress is being made in this area.[96,97]

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REFERENCES

1. P. Buell and J. E. Dunn, Relative impact of smoking and air pollution in lung cancer. *Arch. Environ. Health,* 15 (1967) 291–297.
2. P. Buell, J. E. Dunn, and L. Breslow, Cancer of the lung in Los Angeles type air pollution, prospective study. *Cancer,* 20 (1967) 2139–2147.
3. D. B. Clayson, Chemical and environmental carcinogenesis in man. *European. J. Cancer,* 3 (1967) 405–416.
4. G. C. Buell, Y. Tokiwa, and P. K. Mueller, Lung collagen and elastin denaturation *in vivo* following inhalation of nitrogen dioxide. Presented at the 59th Annual Air Pollution Control Association Meeting, San Francisco, California, June, 1966.
5. G. C. Buell, unpublished observations.
6. D. B. Wetlaufer, Ultraviolet spectra of proteins and amino acids. In, *Advances in Protein Chemistry,* Vol. 17, pp. 303–390, C. B. Anfinsen, Jr., Kenneth Bailey, M. C. Anson, and J. T. Edsall (eds.). Academic Press, Inc., New York, 1960.
7. M. Joly, *A Physico-chemical Approach to the Denaturation of Proteins,* p. 183. Academic Press Inc., New York, 1965.
8. F. M. Sinex and B. Faris, The reaction of tobacco smoke condensate with collagen and elastin. *Abstr. Am. Chem. Soc.,* 135th Meeting, April 5–10, 1959, Boston, Massachusetts.
9. H. Moriyama and and S. Ohashi, *J. Shanghai Sci. Inst.,* Sec. IV, 3 (1938) 161–175; 4, 17–38.
10. P. Rondoni. Some aspects of carcinogenesis. In, *Advances in Cancer Research,* Vol. III, pp. 171–221, J. P. Greenstein and A. Haddow (eds.). Academic Press Inc., New York, 1955.
11. *Ibid.,* p. 210.
12. H. Rubin, Fact and theory about cell surface and carcinogenesis. In, *Major Problems in Biology,* pp. 315–337, M. Locke (ed.). Academic Press, Inc., New York, 1966.
13. D. M. Pace, Effect of ozone on cells *in vitro. Arch. Environ. Health,* 18 (1969) 165–167.

14 E J Ambrose, The surface properties of tumour cells In, *The Biology of Cancer* pp 65–77 E J Ambrose and F J C Roe (eds) D Van Nostrand Co , New York, 1966
15 *Ibid* pp 65 68
16 *Ibid* p 68
17 A E Williams and N A Ratcliffe, Attachment of ascites tumour cells to rat diaphragm as seen by scanning electron microscopy *Nature* 222 (1969) 893 895
18 Letter, Department of the Army, file MEDEC ZHE, dated April 18, 1967
19 R Rossle, Die narben krebse der lungen *Schweiz Med Wochschr* 73 (1943) 1200
20 T Gillman J Penn D Bronks, and M Roux, Abnormal elastic fibers appearance in cutaneous carcinoma, irradiation injuries, and other arterial and degenerative connective tissue lesions in man *Arch Pathol Chicago* 59 (1955) 733–749
21 C Raeburn and H Spencer, Lung scar cancers *Brit J Tuberc Dis Chest* 51 (1957) 237 245
22 J Paul, Metabolic processes in normal and cancer cells In *The Biology of Cancer* pp 52–64, E J Ambrose (ed) D Van Nostrand Co , London 1966
23 J H Peters and E Goetzl, Recovery of greater antigenic reactivity in conformationally altered albumin *J Biol Chem.* 244 (1969) 2068–2074
24 R T Smith, Tumor specific immune mechanisms *New England J Med* 278 (1968) 1207–1214, 1268 1275, 1326–1331
25 L A Zilber, A study of tumor antigens *Acta Unio Intern Contra Cancrum* 15 (1960) 933 939
26 K Habel, Tumor specific antigens In, *Proceedings of the Eighth Canadian Cancer Conference* pp 390 405, J F Morgan (ed) Pergamon Press, Toronto, 1969
27 M A Lappé, Tumour specific transplantation antigens possible origin in pre malignant lesions *Nature* 223 (1969) 82 84
28 R P Barden, Collagen disease and cancer *Radiology* 92 (1969) 972–974
29 R A Good, Genetics and cancer *Nature* 221 (1969) 1195
30 R T Smith, Tumor specific immune mechanisms *New England J Med* 278 (1969) 1207–1214, 1268–1275, 1326 1331
31 H Busch, *The Cancer Cell as a Parasite* p 372 Academic Press, Inc New York, 1962
32 L S Mann and W H Welker, A specific precipitin antiserum for carcinoma protein *Am. J Cancer* 39 (1940) 360–364
33 L S Mann and W H Welker, Further studies of specific precipitin antiserums for the protein of cancer tissue *Cancer Res* 3 (1943) 193 197
34 L S Mann and W H Welker III Relation of proteins of different malignant tissue to each other *Cancer Res* 6 (1946) 625–626
35 P A Gorer, The role of antibodies in immunity to transplanted leukaemia in mice *J Pathol Bacteriol* 54 (1942) 51 65
36 S De Carvalho and L Renerts, Segregation of antigens from human leukemia tumoral cells by fluorocarbon extraction *J Lab Clin Med* 56 (1960) 333–341
37 S De Carvalho, H J Lewis, A J Reid, and J R Ulnick, Immunochromatographic partition of soluble antigens on columns of insoluble diazo-γ globulin *Nature* 204 (1964) 265–266
38 S H Nadler and G E Moore, Immunotherapy of malignant disease *Arch Surg* 99 (1969) 376 381
39 H Harris, O J Miller, G Klein, P Worst, and T Tachibana, Suppression of malignancy by cell fusion *Nature,* 223 (1969) 363–368
40 G C Buell, R Mosley, and P K Mueller, Further evidence for formation of carbonyls in lung tissue following exposure to ozone Presented at 156th ACS Meeting, Atlantic City, New Jersey, September 8–13 1968
41 P S West and W R Todd, *Textbook of Biochemistry* p 160 The Macmillan Co , New York 1955

42. E. N. Trifonov, Y. S. Lazurkin, and M. M. Frank-Kamenetski, Formaldehyde-induced uncoiling of DNA. *Mol. Biol.* (transl. from Russian), 1 (1967) 142–151.
43. B. D. Goldstein and O. J. Balchum, Ozone and lipid peroxidation. *Arch. Environ. Health,* 18 (1969) 631–635.
44. H. V. Thomas, P. K. Mueller, and R. L. Lyman, Lipoperoxidation of lung lipids in rats exposed to nitrogen dioxide. *Science,* 159 (1968) 532–534.
45. C. Little and P. J. O'Brien, The effectiveness of a lipid peroxide in oxidizing protein and non-protein thiols. *Biochem. J.,* 106 (1968) 419–423.
46. W. F. Forbes and J. V. Ramsbottom, Reactivity of trapped free radicals in biological systems. *Nature,* 216 (1967) 913–914.
47. W. T. Raubol and A. L. Tappel, Damage to proteins, enzymes and amino acids by peroxidizing lipids. *Arch. Biochim. Biophys.,* 113 (1966) 5–8.
48. A. L. Tappel, Free-radical lipid peroxidation damage and its inhibition by Vitamin E and selenium. Given at Nutrition Society Symposium, 1964 (published in *Fed. Proc.,* 1964).
49. L. M. Shabad, L. N. Pylev, and T. S. Kolesnichenko, Importance of the deposition of carcinogens for cancer induction in lung tissue. *J. Natl. Cancer Inst.,* 33 (1964) 135–142.
50. Cancerous and normal tissues differ markedly in free-radical, EPR studies. *Chem. Eng. News,* September 6, 1965, p. 23.
51. N. M. Emanuel, A. N. Saprin, V. A. Shabelkin, L. E. Kozlova, and K. E. Krugljakova, Detection and investigation of a new type of ESR signal characteristic of some tumour tissue. *Nature,* 222 (1969) 165–167.
52. H. P. Morris, Studies on the development, biochemistry and biology of experimental hepatoma. In, *Advances in Cancer Research,* Vol. IX, pp. 228–303. Academic Press Inc., New York, 1965.
53. H. U. Boenig, Free radicals: indicators for a unifying concept. *J. Am. Geriatrics Soc.,* 14 (1966) 1211–1220.
54. M. M. K. Nass, Mitochondrial DNA: advances, problems and goals. *Science,* 165 (1969) 25–35.
55. H. E. Bond, J. A. Cooper, II, I. F. Corrington, and J. S. Wood, Microsome associated DNA. *Science,* 165 (1969) 705–706.
56. R. H. Fetner, Chromosome breakage in *Vicia faba* by ozone. *Nature,* 181 (1968) 504–505.
57. H. S. Orlov and E. I. Orlova, A simple method for the determination of deoxyribonucleic acid in animal tissues, *Biochemistry* (USSR), 26 (1961) 722–726.
58. M. Corn, Nonviable particles in air. In, *Air Pollution,* Vol I, 2nd edition, p. 88, A. C. Stern (ed.). Academic Press, Inc., New York, 1968.
59. E. N. Williams, Steroids and cell surfaces. *Biol. Rev. Cambridge Phil. Soc.,* 36 (1961) 368–398.
60. J. Pataki and C. Huggins, Molecular site of substituents of benz(a)anthracene related to carcinogenicity. *Cancer Res.,* 29 (1969) 506–509.
61. R. G. Harvey and M. Halonen, Interaction between carcinogenic hydrocarbons and nucleosides. *Cancer Res.,* 28 (1968) 2183–2187.
62. P. Brookes and P. D. Lawley, Reaction of some mutagenic and carcinogenic compounds with nucleic acids. *J. Cellular Comp. Physiol.,* 64 (1964) 111–128.
63. S. Epstein, N. Ito, L. Merkow, and E. Farber, Cellular analysis of liver carcinogenesis: the induction of large hyperplastic nodules in the liver with 2-fluorenylacetamide or ethionine and some aspects of their morphology and glycogen metabolism. *Cancer Res.,* 27 (1967) 1702–1711.
64. J. H. Weisburger, E. D. Bergmann, and B. Pullman, Carcinogenesis: physicochemical mechanisms. *Science,* 165 (1969) 417–421.

65 J A Di Paola, R L Nelson, and P J Donovan, Sarcoma producing cell lines derived from clones transformed *in vitro* by benz(a)pyrene *Science,* 165 (1969) 917–919
66 R Shapiro, Chemistry of guanine In, *Progress in Nucleic Acid Research and Molecular Biology,* Vol VIII, pp 73 112 J N Davidson and W E Cohn (eds) Academic Press, Inc New York, 1968
67 S Laskin and M Kuschner, Experimental induction of lung cancer 60th Meeting, Air Pollution Control Assoc , Cleveland, June, 1967
68 Glossary of terms In *The Biology of Cancer* pp 231–234 E J Ambrose and F J C Roe (eds) D Van Nostrand Co , London, 1966
69 H V Thomas, P K Mueller, and R Wright, Response of rat lung mast cells to nitrogen dioxide inhalation *J Air Pollution Control Assoc* 17 (1967) 33 35
70 I Danishefsky, E Oppenheimer, O Heritier Watkins, A Bella, Jr and M Willhite, Biochemical changes in the connective tissue pocket surrounding subcutaneously imbedded films *Cancer Res* , 27 (1967) 833–837
71 G Bryson and F Bischoff, Silicate-induced neoplasms *Prog Exptl Tumor Res* 9 (1967) 77–164
72 P Gross, R T P de Treville, E B Tolker, M Kaschak, and M A Babyak, Experimental asbestosis *Arch Environ Health* 15 (1967) 343 355
73 P F Holt, J Mills, and D K Young, Experimental asbestosis with four types of fibers, importance of small particles *Ann N Y Acad Sci* 132 (1968) 87–89
74 C S Sleggs, Clinical aspects of asbestosis in the Northern Cape In, *Proc Pneumo coniosis Conf , Johannesburg,* pp 383–390 Little, Brown and Co , Boston, 1960
75 R C Browne, *The Chemistry and Therapy of Industrial Pulmonary Diseases* p 15 Charles C Thomas, Springfield, Illinois, 1966
76 G S Marks and L W Marass, Changes in the lung lipids of rabbits and guinea pigs exposed to the inhalation of silica dust *Brit J Ind Med* 17 (1960) 31–35
77 M Chvapil, Conflicting hypotheses on experimental silicotic fibrinogenesis new experimental data *Environ Res* , 1 (1967) 89–101
78 J L Kaw and S H Zaidi, Effect of ascorbic acid on pulmonary silicosis of guinea pigs *Arch Environ Health,* 19 (1969) 74–82
79 A M Lee and J F Fraumeni, Jr , Arsenic and respiratory cancer in man *J Natl Cancer Inst* , 42 (1969) 1045–1052
80 R C Browne, *The Chemistry and Therapy of Industrial Pulmonary Diseases* pp 73 77 Charles C Thomas, Springfield, Illinois, 1966
81 *Ibid* , p 81
82 W C Hueper, *Occupational and Environmental Cancer of the Respiratory System,* pp 21–22 Springer-Verlag, New York, 1966
83 R C Browne, *The Chemistry and Therapy of Industrial Pulmonary Diseases* p 65 Charles C Thomas, Springfield, Illinois, 1966
84 A J Vorwald, discussion in *Conference on Etiological and Pathogenetic Factors in Carcinogenesis* p 4 Science Information Bureau, Inc , New York, 1962
85 R C Browne, *The Chemistry and Therapy of Industrial Pulmonary Diseases* p 66 Charles C Thomas, Springfield, Illinois, 1966
86 C P McCord, Beryllium as a sensitizing agent *Ind Med Surg* , 20 (1951) 336–339
87 P T Pugliese, C M Whitlock, B Ernst, and R R Williams, Serological reactions of berylliosis patients to extracts of lung tissues *Arch Environ Health,* 16 (1968) 374–379
88 F J C Roe and M C Lancaster, Natural, metallic and other substances as carcinogens *Brit Med Bull* , 20 (1964) 127–231
89 R C Browne, *The Chemistry and Theory of Industrial Pulmonary Diseases,* pp 78–80 Charles C Thomas, Springfield, Illinois, 1966

90 I Danishefsky, E T Oppenheimer, M Willhite, A P Stout, and M M Fishman, Biochemical changes during carcinogenesis by plastic films *Cancer Res*, 19 (1959) 1234 1238
91 T F Hatch Respiratory dust retention and elimination In, *Proc Pneumoconiosis Conf Johannesburg 1959* Fig 6, p 129 J & A Churchill, Ltd , London 1960, Little, Brown & Co , Boston 1960
92 F J C Roe, Cancer as a disease of the whole organism In, *The Biology of Cancer,* pp 1 32, E J Ambrose and F J C Roe (eds) D Van Nostrand Co , London, 1966
93 V R Potter, Recent trends in cancer biochemistry, the importance of studies on fetal tissue In, *Proceedings of the Eighth Canadian Cancer Conference* Vol VIII, pp 9–30, J F Morgan (ed) Pergamon Press, Toronto, 1969
94 G Haughton and D B Amos, Immunology of carcinogenesis *Cancer Res* 28 (1968) 1839 1848
95 J Monod and F Jacob General conclusions teleonomic mechanisms in cellular metabolism, growth and differentiation *Cold Spring Harbor Symp Quant Biol* 26 (1961) 389–401
96 W S Reznikoff, J H Miller, J G Scaife, and J Beckwith, A mechanism for repressor action *J Mol Biol* 43 (1969) 201 213
97 M S Bretscher, How repressor molecules function *Nature* 217 (1968) 509 511

DISCUSSION

Y. Alarie I'm interested in the technique that you are using to isolate collagen or elastin Did you first take the lung and submit it to sodium hydroxide and ethanol treatment before doing any enzymatic extraction

G. C. Buell: No, I sure didn't

Alarie: Well, I couldn't follow exactly what you said That's why I'm asking again What was the exact procedure for your enzymatic extraction?

Buell: The first thing we do is remove the lung and mince it, then, using sodium chloride, we extract most of the soluble proteins, plus the salt-soluble collagen

The next thing we do is remove the water by alcohol extraction, using two absolute alcohols and two ether-alcohol extractions to get a lipid fraction

After that we are able to grind the tissue into small, uniform powder, so that we have a homogeneous mixture of lipid-free lung powder That's the stuff we use We first extract the polysaccharides with, I think, 1 25% potassium bicarbonate and subject them to a neuraminidase hydrolysis and then to a hyaluronidase hydrolysis, at which point we have nothing left but collagen and plastin By subjecting part of that to collagenase, we wind up with elastin By subjecting the other part to elastase, we are left only with the collagen

PULMONARY CELL KINETICS AFTER EXPOSURE TO CIGARETTE SMOKE

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ABSTRACT

A group of 26 male hamsters was exposed to the smoke of four cigarettes every 6 hr for 48 hr The animals were killed serially during the exposure period and for 72 hr after the end of exposure Counts of labeled cells in airways and alveoli after a single dose of ^{3}H thymidine were compared with counts from a group of normal hamsters After the first exposure, increased labeling was found in bronchi, bronchioles, and alveoli Thereafter, labeling counts of bronchi and bronchioles were normal In alveolar areas three additional peaks of increased labeling were found at 40 hr of exposure, at 24 hr of recovery, and 72 hr of recovery

The first aim of this study was to determine whether or not there was a demonstrable response of alveolar, bronchial, and bronchiolar cells to an inhalation exposure to a very low dose of cigarette smoke To this end, hamsters were exposed to cigarette smoke introduced into an exposure chamber, and cellular changes were monitored by autoradiography after pulse labeling with ^{3}H-thymidine The experiment was designed so that the time sequence of labeling was followed during exposure to cigarette smoke and during a recovery period The results were compared to those found in a group of hamsters not exposed to cigarette smoke

The second aim was to compare the pattern of labeling response with that previously found after hamsters were exposed to oxygen for 48 hr at a partial pressure of 555 mm Hg Thus all factors of the present experiment were kept as similar as possible to those of the previous oxygen study

MATERIALS AND METHODS

All animals used in this study were 3-month-old male golden hamsters The 30 hamsters in Group 1 were kept in filtered, air-conditioned holding chambers

Group 2 comprised 26 hamsters exposed to cigarette smoke. Half of the animals in Group 1 were killed during the middle of their light period, under normal light conditions (32 foot candles). The other half of the animals in Group 1 were killed during the middle of their dark period, at night when the lights were off (less than 1 foot candle of available light). This was done to determine whether light-dark cycles have an effect on labeling counts.

All animals were killed by administration of chloroform. One hour before sacrifice each animal was given 500 μCi of ^{3}H-thymidine (4 μCi/g body weight) intraperitoneally. Fifteen minutes later 1000 units of heparin was given intraperitoneally so that vascular perfusion fixation could be performed. After deflation pressure volume relations of the lungs were measured, fixation was carried out by perfusion through the right ventricle with buffered 0.17 *M* glutaraldehyde. Frontal sections of the whole lung were prepared for autoradiography.

Autoradiography made use of NTB2 emulsion (Kodak). After the emulsion had been exposed to the tissue for 2 weeks, slides were developed with D-19 (Kodak) and stained with hematoxylin and eosin.

Labeled cells were counted by a microscopic projection technique. Areas to be counted were selected by use of a 2.5X objective, so the degree of labeling was unknown when the area to be counted was chosen. A 32X objective was then substituted, and the labeled cells were counted. A switch back to the 2.5X objective allowed the next nonoverlapping field to be chosen.

Alveolar areas were projected onto a screen calibrated so that a circle represented 10^5 μ^2 of tissue when the 32X objective was used. Ten such areas (10^6 μ^2) were then the basic unit of the labeling count. For each right and left lung three different alveolar areas were selected: subpleural, peribronchiolar, and midzonal. Five million square microns of tissue were counted in a shell of tissue just beneath the pleura, the subpleural zone. The peribronchiolar zone consisted of alveoli around terminal bronchioles, and three million square microns of tissue were counted in this region. The midzone consisted of alveolar areas between the subpleural and peribronchiolar zones. Five million square microns of tissue were counted in this zone.

Counts of bronchi and bronchioles were done by measuring the length of their projected image with a map-distance measuring device. Six thousand microns of length were counted for airways of three sizes: (1) those greater than 400 μ minimal diameter; (2) those between 200 and 400 μ minimal diameter; and (3) those less than 200 μ minimal diameter.

Hamsters were exposed to cigarette smoke in a cubical chamber having a volume of 62 liters; an airflow of 594 liters/min was maintained through the chamber. Cigarettes were smoked by a peristaltic pump with a flow of 4 liters/min. Smoke pulled through the cigarette was pumped through a venturi opening into the inflow of air into the chamber.

The mass concentration of particles in the diluted cigarette smoke that flowed through the chamber was measured with a forward-light-scattering

aerosol photometer. The photometer was calibrated by weighing the mass of the particles in the sample passing through the photometer. The particles were collected with a molecular filter. The output from the photometer was recorded with a strip chart recorder so that the peak and average concentrations were calculated.

Group 2 hamsters were exposed to the smoke of four cigarettes every 6 hr for 48 hr. Cigarettes were a standard 70-mm unfiltered brand (Camel). Each cigarette was allowed to burn for 30 sec, then removed from the holder. One and a half minutes elapsed between the time that one cigarette was completely smoked and the next one lit. Since one-half minute was required to wash cigarette smoke out of the exposure chamber, animals were exposed to 1 min of smoke from each cigarette. One exposure to four cigarettes required only 7 min. There were nine such exposures over a 48-hr period, giving a total of 36 cigarettes and 36 min of total exposure. The recovery period after the end of exposure was 72 hr.

Pairs of animals were killed at the following times after the beginning of the exposure: 8, 14, 20, 26, 32, 40, and 50 hr. During recovery, pairs of animals were killed at 6, 12, 24, 36, 48, and 72 hr after the end of the exposure.

RESULTS

Group 1: Normal Hamsters

Separate labeling counts were made for the right and left lungs, as well as for each of the three alveolar areas and each of the three differently sized airways. For the subgroups killed during the light and dark periods, the means, the standard deviations, and the significance of difference of the means were calculated. There were no significant differences between the left and right lungs, and none for any counts between hamsters killed during their light period and those killed in the dark.

The labeling counts for airways greater than 400 μ minimal diameter, those of 200–400 μ minimal diameter, and those less than 200 μ minimal diameter were not significantly different. The average number of labeled cells per 1000 μ length was 0.16, the standard deviation was 0.15, and the upper limit at the 95% confidence level was 0.46 labeled cells per 1000 μ length.

Comparison of the subpleural, peribronchiolar and midzonal alveolar areas revealed no statistical differences between these three areas. The average number of labeled cells per million square microns was 3.1, the standard deviation was 1.7, and the upper limit at the 95% confidence level was 6.5.

Group 2: Hamsters Exposed to Cigarette Smoke

The deflation pressure volume relations for all animals were normal except for one animal killed 24 hr after the beginning of the recovery period. In this animal the volume values at the lower pressures were slightly lower than normal.

The time course of the labeling counts was the same for all three differently sized airways. High values were observed eight hr after the beginning of the exposure to cigarette smoke. Thereafter all values were normal. The following differences were found in the height of the 8-hr exposure peaks: bronchioles with less than 200 μ minimal diameter had 0.71 labeled cells per 1000 μ length; those with 200–400 μ minimal diameter had 0.63 labeled cells per 1000 μ length, and airways greater than 400 μ minimal diameter had 0.47 labeled cells per 1000 μ.

The subpleural, peribronchiolar, and midzonal alveolar counts not only showed exactly the same time course but also were extremely close to the same value at all sacrifice intervals. The alveolar counts (Fig. 1) were very high 8 hr after the beginning of exposure, even though these animals had been exposed to a very dilute concentration of smoke from only four cigarettes. After this initial high peak, the labeling counts became lower but remained elevated at 14, 20, and 26 hr of exposure. At 32 hr of exposure the counts were just within the upper range of normal. After 40 hr a second peak of increased labeling was found, but it was not as high as the first peak. The labeling counts observed after 50 hr of exposure were within normal and remained so at 6 and 12 hr of

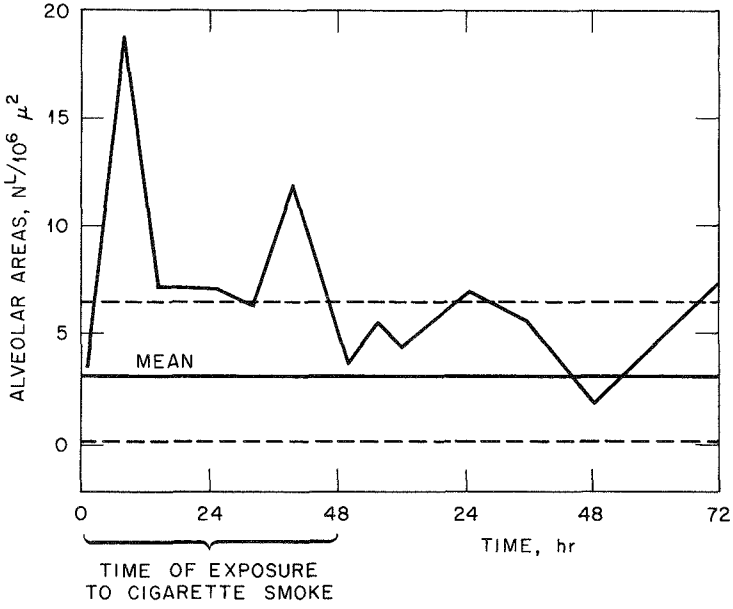

Fig. 1 – Labeling response of alveolar areas after exposure to cigarette smoke. The mean value (3.1) found in animals breathing normal air is represented by the solid horizontal line. The upper limit, which is two standard deviations larger than the mean (6.5), is represented by the upper dashed horizontal line. The lower dashed line indicates that the lower limit of the range approaches zero. N^L represents the number of labeled alveolar cells.

recovery A third low peak was seen at 24 hr of recovery followed by normal values at 36 and 48 hr At the end of the recovery period (at 72 hr) a final, slight elevation of labeling counts was found

These findings must be considered in relation to the cigarette exposure with which they are associated The large flow (594 liters/min) of air through the small-volume (62 liters) exposure chamber reached 99% concentration in only 30 sec This was also the time required to wash smoke out of the chamber These observations confirm Silver's equation [1] The lag time from the start of lighting a cigarette to the recorder response of the photometer was 4 sec Sampling at different parts of the chamber gave the same results It seemed, therefore, that this system gave a reasonable measurement of the transient changes during cigarette smoking and chamber washing The concentration of cigarette smoke rose rapidly during the initial 10 sec of burning, then gave a slowly rising plateau Upon completion of the burn the concentration fell rapidly for 10 sec, then slowly returned to zero over the next 20 sec The peak concentrations of cigarette smoke showed a mean of 127 μg/liter, a standard deviation of 22 μg/liter, and a range of 84–171 μg/liter The average concentration, as determined by weight, gave a mean of 58 μg/liter, a standard deviation of 13 μg/liter and a range of 31 84 μg/liter These values were confirmed by determination of the areas under the curve for each cigarette smoked

From these values the exposure of the animals may be calculated by using Guyton's equation[2] to derive a minute volume of 0 08 liter/min If exposure is concentration in μg/liter multiplied by time of exposure in minutes, multiplied by minute ventilation in liters/min, the dimensions other than μg cancel Substitution gave an exposure of 4 6 μg per cigarette, of 18 4 μg for four cigarettes, and 166 μg for the total nine exposures This exposure would be equivalent to one of 3 5 μg/hr

Exposures, expressed in terms of kg body weight, were 35 μg/kg for each cigarette, 138 μg/kg for each exposure, and 1 2 mg/kg for all exposures

The final calculation of exposure took into account the alveolar retention factor K, which is 1 for gases and less for particles When a cyclone impactor that removed 100% of the particles greater than 10 μ was used, 91% of the mass was still deposited as it would be in lung Multiplying the exposure per kilogram by 91 gave an alveolar exposure of 35 μg per cigarette per kilogram, one of 139 μg for four cigarettes, and a total alveolar exposure of 1 1 mg/kg

DISCUSSION

To avoid any possible effect of the estrus cycle on the labeling of pulmonary cells, only male hamsters were used in these experiments Since no differences were found between the labeling counts of animals in their dark period and those of animals in their light period, this variable probably does not have to be controlled in future inhalation exposures

The demonstration of no significant differences of labeling counts between the left and right lungs, between airways of three different sizes, and between three different alveolar areas simplifies recall of normal values, enables larger samples of tissue to be counted, and provides a basis for localizing inhomogeneous changes. Since the low range of labeling would calculate to be less than zero, an impossibility, the normal group shows conclusively that labeling values less than normal cannot be determined. Only values larger than normal are observable.

By calculating the third and fourth moments for alveolar areas, the distribution was slightly shifted to the left and the peak of the normal distribution curve was slightly blunted. Individual counts tended to cluster in some fields and be absent in others. One possible explanation for this observation might be that inhaled environmental agents give rise to a low labeling count in normal animals. If such agents were unevenly distributed, focal areas of increased labeling could result.

In Group 2 (hamsters exposed to cigarette smoke), the immediate labeling response of airways and alveolar areas is much different than had been seen previously with high oxygen exposure.[3] With high oxygen exposure there was a lag of 24 hr before increased labeling was observed. Such a rapid, large response of cells located from large (400 μ minimal diameter) bronchi to subpleural alveoli after an exposure to only 18 μg of cigarette smoke is striking.

The failure of airway epithelium to subsequently follow the same repeated peaks and valleys as seen in alveolar areas may be a result of greater particle deposition in alveolar areas, or of greater effects of gases of cigarette smoke at the alveolar areas, or of different cellular responses.

The repeated peaks of alveolar areas during exposure are similar to those described in strippling human skin.[4] This pattern was not found after exposure to high oxygen. Moreover, these peaks are probably not a result of intermittant exposure to cigarette smoke. The period of 6 hr should be less than the time of DNA synthesis, so that the exposure is like a continuous one.

The peaks at 24 and 72 hr of recovery are important in relation to the total exposure of 166 μg. If smoked directly instead of being diluted 145 times by air flowing through a chamber, the smoke from one cigarette would have an average concentration of 8,265 μg/liter, and exposure to one cigarette would be 667 μg.

The occurrence of peaks of labeling during recovery is yet another difference between the findings of cigarette exposure and high oxygen exposure. Although increased labeling persisted for 24 hr of recovery from high oxygen, it did not fluctuate or show the late (72 hr) response.

The fluctuations in labeling counts during exposure and recovery dictates the design of inhalation exposures. It would be impossible to compare a normal group of animals and an experimental group sacrificed at any one time: Either normal or elevated counts could be observed depending upon the time of sacrifice.

The occurrence of marked changes in labeling in the absence of alteration of deflation pressure volume curves shows that cellular alterations are demonstrable in the absence of a change in a physical property of the lining layer of the lung.

In a steady-state condition with a random distribution of the times of the events of the cell cycle, the ratio of $Ts/T_{G1} + T_{G2} + T_M$ determines the labeling count. Any condition which increased the spread of the distribution of these times would alter the labeling counts. Since labeling counts cannot diminish below normal, only normal or elevated counts would result from an increased range of time distributions. Baserga and Wiebel[5] reviewed the great variation in the durations of events of the cell cycle in mammals and the findings in some tumors. In some tumors the Ts was prolonged, the T_{G1} was diminished markedly, and the T_{G2} was lengthened. The length of the cell cycle time was not necessarily shorter than normal. Such changes are consistent with the observations of this study.

The repeated peaks of increased labeling suggested a periodicity of events. Such periodicity could result from partial synchrony of cell populations. Lengthening of Ts or shortening of T_{G1} (or T_{G2}), or both changes occurring at regular intervals, would give periodic peaks. Persistence of repeated peaks is evidence for a continuation of altered events of the cell cycle of a population of cells.

The magnitude of the initial peak strongly suggested that there was a shortening of the T_{G1} of a large number of alveolar cells. The continued elevation of labeling counts for 12 hr after the initial peak could have been a result of lengthening of Ts. The repeated peaks, their periodicity, and their persistence could have been a result of the changes just described.

The pattern of the labeling response to cigarette smoke, a mixture of a large number of chemical carcinogens, was significantly different from that of the response to high oxygen, a noncarcinogenic agent. Cigarette smoke gave an immediate response, followed by repeated peaks of labeling, and a continuation of labeling peaks for the duration of recovery. None of these findings were present after exposure to high oxygen.

Acknowledgements

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REFERENCES

1. S. D. Silver, Constant flow gassing chambers: Principles influencing design and operation. *J. Lab. Clin. Med.*, 31 (1946) 1153–1161.
2. A. C. Guyton, Measurement of respiratory volumes of laboratory animals. *Am. J. Physiol.*, 150 (1947) 70–77.
3. H. G. Boren and E. L. DuBrow, Autoradiographic study of pulmonary response to oxygen. *Am. J. Pathol.*, submitted for publication.

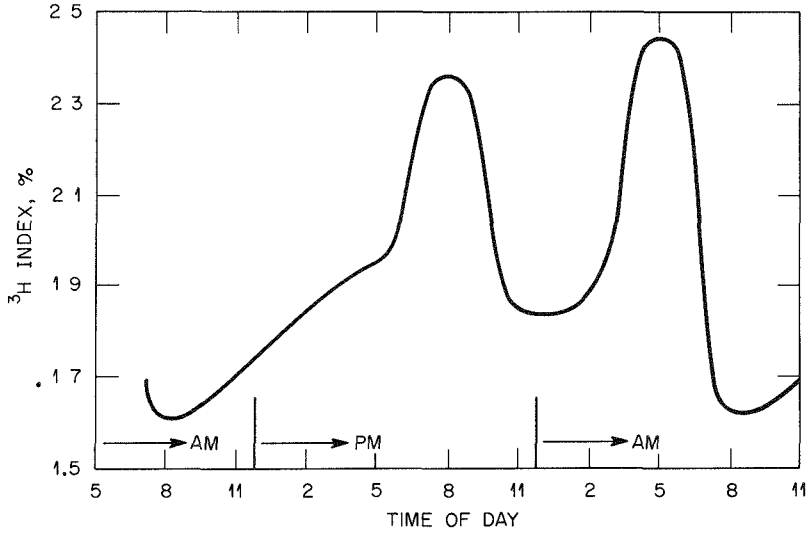
4 W L Epstein and D J Sullivan, Epidermal mitotic activity in wounded human skin In, *Biology of Skin,* Vol V, pp 68–75, Wm Montagna and R E. Billingham (eds) Macmillian Co , New York, 1964
5 R Baserga and F Wiebel, The cell cycle of mammalian cells In, *International Review of Experimental Pathology,* pp 1–27, G W Richter and M A Epstein (eds) Academic Press, New York, 1969

DISCUSSION

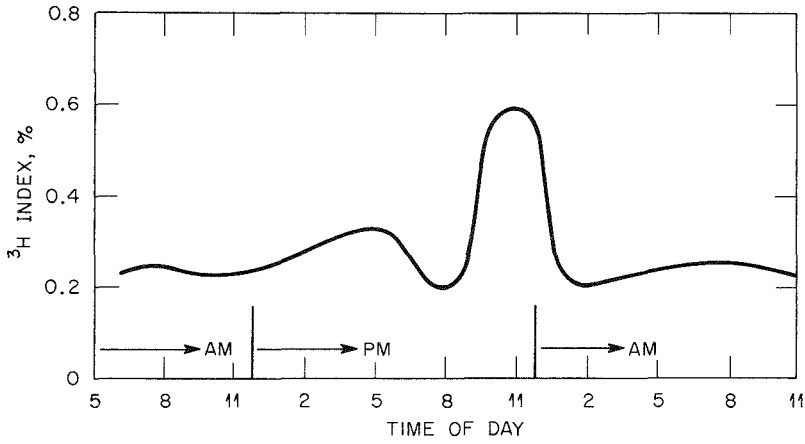
M. C. Battigelli: I wonder if Dr Boren would care to comment on the criteria used in identifying the type B alveolar cells, and, too, if he used a staining technique other than H & E to study alveolar wall cellular population

H. G. Boren: No, we used only H & E The criterion for type B cells in the light microscope was the presence of spherical nuclei with a large amount of cytoplasm, with inclusions of vesicles Using the profusion fixation technique, we washed the blood out of the thin capillaries, so that the patency of the capillaries was maintained and we could identify the type B cells with 40X objectives

W. Dontenwill: One comment We measured the labeling index during light and dark periods This [Discussion Fig 1] is the labeling index in the skin for 1 day and this [Discussion Fig 2] is the labeling index in the lung for 1 day You see in the skin two peaks at the time of the highest locomotor activity, and you see in the lung, in the bronchi, one peak in the highest locomotor activity The method for measuring this is different from yours It's a method published by



Discussion fig 1 – Summation curve of ^{3}H labeling in hamster epidermis See discussion for details



Discussion fig. 2 – Summation curve of ^{3}H labeling in bronchial epithelium of hamster. See discussion for details.

Oehlert and a co-worker [W. O. Oehlert and D. Grimm, *Z. Krebsforsch.*, 15 (1966) 14–24.].

And one question: Have you measured the labeling index after exposure to carbon monoxide alone?

Boren: No, we've not measured the labeling index after carbon monoxide exposure. On the basis of studies such as yours, we expected to see differences in light-dark cycles of pulmonary cells. We were surprised that we did not.

D. Hoffmann: Seeing your study and having an idea about autoradiography, I realize that this work must have demanded great efforts in time and patience. People who may be interested in going into this area may be frightened to start such a study because of all the counting. What are your ideas about doing this with liquid scintillation counting? This may be possible by separation of the cell constituents and then by counting their beta activities. In this case one could use not only ^{3}H-thymidine but also ^{14}C-labeled thymidine. In my opinion, based on other studies, this process would significantly reduce the experimental variation, which we now have to a significant degree. Furthermore, this process wouldn't be so time-consuming. However, we haven't done it, and I would be glad to get your ideas on this.

Boren: Scintillation counting might work on cells washed out of the lung. The disadvantage of using the liquid scintillation counting would be the loss of morphologic identification of cell populations. With the projection technique we can teach technicians to do the counts very rapidly.

C. Loosli: What is the thickness of the section?

Boren: The thickness is 5 μ. If you use data published by Feinegeden which indicate that about 99% of the beta rays don't penetrate beyond 2.5 μ, you are really counting cells labeled at the surface.

K. H. Kilburn: I have two questions. The first is: Have you looked at any animals that had had previous acquaintance with cigarette smoke? One wonders if the response would be the same after they had had previous exposure.

My second question is: You presented data based entirely on changes in the parenchyma of the lung and including only small airways. What about cellular changes in the trachea and large bronchi? Are there any changes in the common sites at which carcinoma develops?

Boren: I don't know if the animals were exposed to cigarette smoke before they got to us or not. I know that they were kept within our holding chambers, where they breathed only tempered, filtered air for 2 weeks before they were put into an experiment. We have not specifically designed a study to see what happens with tolerance, acclimatization, or adaptation to cigarette smoke.

In hamsters, the largest bronchi had a minimal diameter of over 400 μ.

M. Kuschner: In regard to interpretation of the data, you suggested that the lag period might relate to the time needed to turn off the functional genes and the time required to turn on genes concerned with growth and replication. I think this lag is common to other systems, as you pointed out. In some well-described ones, such as the liver, the lag time was the time required for RNA synthesis and protein systhesis to supply the necessary enzymes for replication. As a matter of fact, in the liver, albumin production goes on even during replication.

The second question I have relates to an interpretation which you seem to carefully avoid and, I guess, properly; that is, does labeling imply replication? There seems to be more than a hint, now, that there can be a type of DNA synthesis that is not necessarily concerned with cell turnover; and in that regard I wondered whether you had any correlation between mitotic count and labeling? You hinted at some discrepancies between the results of generation times calculated by mitotic counts and labeling procedures.

Boren: We can't do mitotic counts, simply because hamsters are genetically resistant to colchicine. One-third of all the slides presented this morning went through the sequence of 0.1 *M* sodium pyrophosphate at 4°C for 10 min, then through the 5% TCA at 4°C for 20 min. We saw no wash-out of label from this. This was an indication that our labeling was of the DNA.

T. Crocker: I might respond to Dr. Hoffmann's question regarding the counting of replicative activity in lung tissue, which does become an important issue, technically, in terms of the labor involved in cell counts. We have measured labeling from the ribbon of serial sections in the fashion that you suggest and by additional methods. We have taken sections in parallel with

autoradiographic sections and exposed certain of them to Hyamine overnight in an incubated shaker. Digestion with Hyamine freed protein and nucleic acids from the paraffin of the section. The paraffin was then dissolved by the toluene-based fluor, and scintillation counting was performed. In parallel histologic sections, tissue areas were measured with an eye-piece micrometer so that the scintillation counts could be correlated with the unit areas of tissue represented by the section.

In addition to autoradiographic counts of labeled and total nuclei and the scintillation counts per unit area of tissue, DNA was extracted from homogenized lung by the method of Ogur and Rosen, and the specific activity of DNA determined. The three methods for estimation of replication were, therefore, the specific activity of DNA, scintillation counting per unit area of tissue in sections, and the proportion of labeled cells in an autoradiograph.

These measurements correlated well when applied to a simple model. Hamster and mouse lungs were removed for study at frequent intervals during the period between zero and 14 days of age. During this period a very rapid growth of lung volume and weight occurs, accompanied by proliferation of cells in all portions of the lung. All three methods showed proportionately the same degree of increase in labeling activity. Thus it is technically possible to use simpler methods for estimation of overall proliferative events in a tissue.

J. Kleinerman: I would like to insert a word of caution about interpreting light microscopic autoradiographs. There are several types of cells both in the alveolar structures and in the bronchioles, and with the light autoradiographic method, it's not possible to distinguish mitotic activity, or labeling, within these various cell types. In the bronchioles there are secretory and ciliated cells, and even brush cells; and in the alveoli there are type I, or type A, cells and type II cells. I think that there is some question as to how definitively one can distinguish type II cells by light autoradiography. I would like to ask you if in your counting you included the detached cells which are considered to be macrophages.

Boren: No, we did not. We didn't have any significant number of detached cells. These were all cells in the alveolar walls.

I agree entirely with you on the problem of identifying compartments.

C. Kensler: In your discussion of cigarette smoke administration, you mentioned that you achieved, I think, maximal concentration of 125-μg per liter. These were micrograms of what? Is this wet weight of particulate matter, or what?

Boren: These were particles that were collected upon a molecular filter from cigarette smoke.

Kensler: What I wondered is Can you express this in another way? Did this represent a 1 to 500, or 1 to 1000, or 1 to what dilution of cigarette smoke that these animals were exposed to?

Boren: 1 to 150

A. B. Reiskin: I am concerned about your ability to identify specific cell types as being labeled or unlabeled, if you are working in thick sections, although your remarks about the ability to detect label on a surface may be quite right

Second, you can do collections of miotic figures in hamsters with colchicine, although you have to go to much higher doses than in other rodents However, I don't think it will provide a simple answer There are many problems associated with mitotic collections which are often ignored You can also use vinblastin and a variety of other compounds

Boren: We tried that but failed

Reiskin: Well, I don't know why it wouldn't work in the lung There are certainly several references in the literature reporting success in other hamster tissues

Did you say that you used 4 μCi/g of ^{3}H-thymidine?

Boren: Right

Reiskin: That sounds like a rather high dose, which might produce toxicity I also wonder if you could comment on the possibility that you are looking at two different things when you compare the response to cigarette smoke and the response to oxygen The oxygen may be producing a toxic effect while the cigarette smoke containing nicotine, carbon monoxide, etc , may be altering the blood flow in the animal, which could change the labeling pattern in the lungs independent of any toxicity

Boren: We examined doses from 0 5 μCi up to 6 μCi very carefully, and we saw no differences We used 4 μCi because it gave us a 2-week dark time

Also, as I mentioned, we are giving ^{3}H after our exposure, so it can't influence our results

In terms of oxygen toxicity, we chose our exposures on the basis of Howard Karsner's work showing pulmonary edema to be produced in 72 hr The fact that we did not see pulmonary edema except in one animal showed that we were just below the range where we produced pulmonary edema This was confirmed by deflation pressure volume curves

J. B. Little. I would like to add, in relation to the question of autoradiographic scoring of labeled cells versus the biochemical separation and counting of radioactivity in DNA, that actually we are measuring two quite different things by these techniques In the case of autoradiography we are

scoring the fraction of cells which are in the DNA synthetic (S) phase of the cell cycle at a given time, whereas by the radioactivity counting technique we are simply measuring the overall rate of DNA synthesis in the tissue

In the case where we are trying to determine the response to an inhaled carcinogen, therefore, the two parameters we are measuring may be quite different In the case of autoradiography we are actually scoring the change in the number of cells that are in DNA synthesis

Radioactivity measurements will reflect both changes in the number of synthesizing cells as well as in the rate of DNA synthesis in individual cells, or the duration of the S phase, but cannot differentiate between the two effects As Dr Crocker pointed out, however, changes in uptake of radioactivity may in some systems be due entirely to changes in the fraction of cells in DNA synthesis

Loosli: Are there further questions for Dr Boren? If not, Dr Hoffmann would like to direct a question to Dr Crocker

Hoffmann: Dr Crocker, in your studies in which you separated cell constituents and then counted, did you find any evidence of a different incorporation rate of thymidine into, let's say, DNA in the mitochondria and the DNA in the nucleus?

Crocker: I'm sorry, we may have a misunderstanding DNA was extracted from homogenized lung in which are first removed lipids, followed by extraction of RNA before final recovery of DNA The specific activity of DNA was determined from scintillation counts per milligram of DNA, as measured by UV spectrophotometry

INFLUENCE OF NITROGEN DIOXIDE ON RESISTANCE TO RESPIRATORY INFECTIONS

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ABSTRACT

Studies reported from this laboratory suggest that reduction in resistance to bacterial pneumonia is the most sensitive indicator of the biological effects of nitrogen dioxide (NO_2) — Exposure of Swiss albino mice or of inbred mice to 3 5 ppm of NO_2 for 2 hr resulted in a significant increase in mortality from respiratory challenge with airborne *Klebsiella pneumoniae* Continuous exposures to 0 5 ppm of NO_2 for 3 months also resulted in decreased resistance to bacterial pneumonia, while intermittent exposures to 0 5 ppm NO_2 for 6 or 18 hr per day produced similar effect after 6 months The various conditions of exposure to NO_2 reduced the capacity of the animals to clear viable bacteria from the lung In addition, chronic exposures resulted in expansion of lung alveoli, and the lesions appeared to be consistent with the development of early emphysema — Recent data on acute and chronic exposure of squirrel monkeys to 5 to 50 ppm of NO_2 suggest that their resistance to infection initiated by respiratory challenge with airborne *K pneumoniae* or influenza virus is also significantly reduced

Air pollutants exert their effect by contact with the membranous surfaces of the body, which, because of their high absorptive capacity, are particularly sensitive to injury The extent of damage is related to the duration of exposure, concentration of the pollutant and, to a very large degree, to its solubility Upon inhalation, gases such as sulfur dioxide and nitrogen dioxide (NO_2), are differentially dissolved in the aqueous phase of the respiratory tract Sulfur dioxide, being relatively soluble in water, is rapidly dissolved in the upper portion of the airway The less soluble NO_2 reaches deeper into the lungs and exerts its irritating effect in the lower portion of the respiratory tract Such irritation can lead to alterations in ciliary movement, production of mucus, and activity of alveolar phagocytes Upon more severe exposure, pulmonary edema can develop Since these functions represent natural defense mechanisms against

respiratory infections, the effects of NO_2 on the respiratory system are of special interest from the standpoint of infection

This paper reviews data obtained in our laboratories on the effects of NO_2 on resistance of experimental animals to bacterial pneumonia and influenza The effects of special interest were (a) alterations in mortality rate and survival time and (b) the ability of the animal host to eliminate inhaled microorganisms from the lung

METHODS

The methods used for exposing experimental animals to NO_2 have been reported earlier [1,2] Briefly, to provide similar environments for the control and experimental animals, pairs of identical chambers were used, namely, small Plexiglas enclosures for acute exposure and walk-in chambers for chronic exposure To establish the NO_2 environment in the chambers small amounts of 99 5% minimum purity NO_2 were passed into a glass mixing chamber where the gas was diluted and mixed with air that was filtered through conventional and activated charcoal filters The air-NO_2 mixture was then introduced into the exposure chamber, providing about 20 air changes per hour To verify the homogeneity of NO_2 in the chamber, air samples were continuously monitored by a Mast gas analyzer

The infectious agents used in the studies were *Klebsiella pneumoniae,* type A-D, and influenza virus, strain A/PR-8 Details of techniques used for growth, dissemination, and respiratory challenge were previously described [3,4] The microorganisms, grown in appropriate media, were disseminated by a modified University of Chicago Toxicity Laboratory type nebulizer to produce an aerosol consisting mainly of particles in the 1- to 5-μ range [3] Mice and hamsters were challenged with the infectious aerosols by the respiratory route in suitable chambers Squirrel monkeys were infected intratracheally by instillation In all infectious challenges two groups of animals were used simultaneously, namely, those exposed to NO_2 and controls not exposed to the gas An additional group of animals was included to determine the effects of NO_2 *per se* In studying the predisposing effects of NO_2, groups of 10 mice and 6 hamsters were exposed to the pollutant first and then, at intervals, were challenged with the infectious agents

The quantitative data are reported as (a) excess mortalities of experimental animals over those of control animals challenged with the infectious agent alone, (b) mean survival time based on an overall survival of 14 days, and (c) rate of clearance of viable microorganisms from lungs Whenever applicable, the data were compared by a standard t test for paired experiments or by analysis of variance techniques To determine the significance of the observed differences among treatment means the 5% probability level was used

RESULTS

Bacterial Pneumonia

Effect of acute exposure. In this paper the phrase *acute exposure* means a 2-hr exposure of the animal to various concentrations of NO_2 ranging from 1.5 to 65 ppm. Throughout the experiments mortalities were not observed in mice, hamsters, or squirrel monkeys exposed to NO_2 alone. Thus the reported excess mortalities reflect increases in death rates of animals subjected to the combined stress (infectious challenge and NO_2 exposure) over the death rates of animals challenged with the infectious agent alone.

Figure 1 illustrates the excess mortalities in Swiss albino mice exposed to various concentrations of NO_2 followed within 1 hr by respiratory challenge. The excess mortalities in groups of mice exposed to 1.5 or 2.5 ppm of NO_2 were not significant, while those in mice exposed to NO_2 concentration of 3.5 ppm or higher were highly significant (shaded bars). Thus the data suggested a threshold level of about 3 ppm at which exposure to NO_2 resulted in a significantly reduced resistance to bacterial pneumonia.[5] This decrease, however, was not

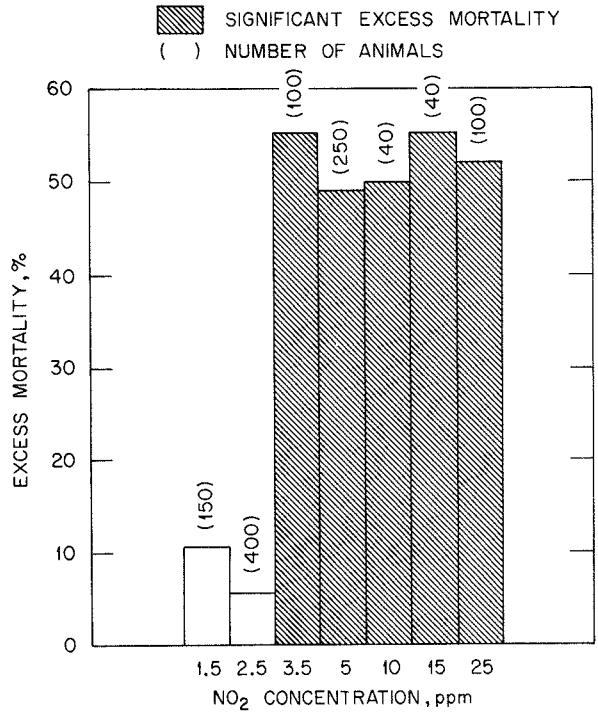

Fig. 1 – Excess mortalities in Swiss albino mice after 2-hr exposure to NO_2 and challenge with *K. pneumoniae.* Significant excess mortality is shaded; number of animals is given in parentheses.

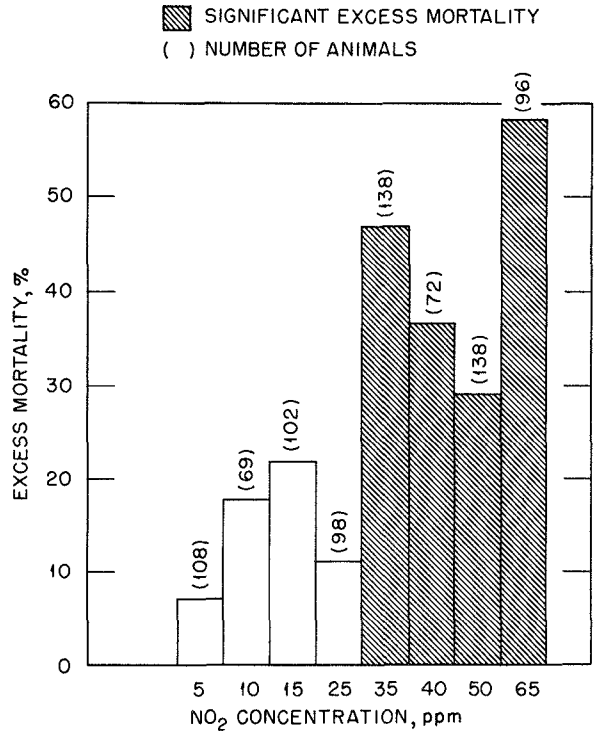

Fig. 2 – Excess mortalities in hamsters after 2-hr exposure to NO_2 and challenge with *K. pneumoniae.*

permanent. When the time interval between termination of the NO_2 exposure and the infectious challenge was extended from 1 to 27 hr, allowing for recovery from the NO_2 exposure stress, the death rates in the experimental groups were almost identical to those in the control groups. Interestingly, the extent or persistence of the effect of NO_2 were not influenced by the progressive increase in concentration of the gas above 3.5 ppm.

Excess mortalities in hamsters exposed for 2 hr to NO_2 in concentrations ranging from 5 to 65 ppm are shown in Fig. 2. As can be seen, exposure to NO_2 from 5 to 25 ppm resulted in some excess mortalities, but the increases were not significant. Exposures to NO_2 of 35 ppm or higher resulted in a significant increase in the death rates (shaded bars). In hamsters the effective threshold level, at which NO_2 significantly reduced the resistance to bacterial pneumonia, appeared to be approximately 30 ppm. Exposures to NO_2 above this level did not result in an incremental increase of mortalities.

The third species of animals used in the acute exposure studies was the squirrel monkey. An intratracheal dose of *K. pneumoniae* estimated at 10^4 to 10^5 organisms did not kill the control monkeys, and only one monkey died

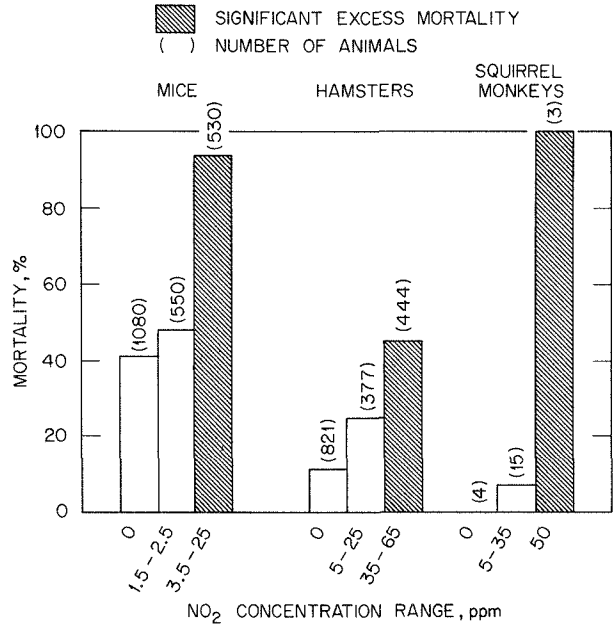

Fig. 3 – Effects of 2-hr acute exposure to NO_2 and challenge with *K. pneumoniae* on mortality of mice, hamsters, and squirrel monkeys.

among 15 exposed to 5 to 35 ppm of NO_2. After exposure to 50 ppm of NO_2, three out of three monkeys died.

The actual death rates in the three species of animals and the numbers of animals used to obtain these means are shown in Fig. 3. Within each animal species, the mortality data were subdivided into three groups. The first was data from the control group of animals challenged with the infectious agent but not exposed to the gas. The second group respresented data of animals challenged with the infectious agent and exposed to concentrations of NO_2 which did not significantly enhance the mortality. The third group was data of animals at NO_2 levels at which the enhancement in mortality was significant.

The mortality data of control animals indicate the range of natural resistance to bacterial pneumonia in different species of animals. The respective death rates in control mice, hamsters, and monkeys were 41%, 11%, and 0%. The respiratory dose for monkeys and hamsters was approximately 10^5 organisms, a dose which repeatedly killed all Swiss albino mice. Differences in natural resistance to bacterial pneumonia were also observed in studies of the effects of NO_2 in five strains of mice. The BDF_1 and C57BL mice showed significantly higher natural resistance to the infection than the BALB, LAF, or Swiss albino mice. However, excess mortalities ranging from 8 to 25% were observed in all five strains of mice after exposure to 5 ppm of NO_2 and the infectious challenge.

The increased mortalities from the acute exposure to NO_2 were persistently paralleled by significant decreases in the mean survival time, which was estimated on the basis of a 14-day overall holding period after the infectious challenge [5] The mean survival time of Swiss albino mice challenged with *K pneumoniae* alone ranged from 10 to 11 5 days After exposure to the combined stresses of infectious challenge and exposure to up to 2 5 ppm of NO_2 the survival time did not change significantly Upon exposure to concentrations above 3 5 ppm of NO_2 the mean survival time decreased significantly and ranged from 4 7 to 6 6 days Similarly, the mean survival time of hamsters decreased from about 13 days for controls to about 5 days for experimental animals exposed to 25 or 35 ppm of NO_2

Autopsy of mice that died after the infectious challenge revealed a high incidence of purulent exudate in the pleural cavities The lungs, usually consolidated and reddish brown, often contained white plaques resembling colonies of *K pneumoniae* The lungs of mice exposed to 3 5 to 25 ppm of NO_2 showed varied degrees of congestion and dilatation of veins and capillaries Lungs of mice exposed to 1 5 or 2 5 ppm of NO_2 were essentially free of pathological changes

Autopsy of squirrel monkeys that died after the combined exposures disclosed a massive infection of the lung *K pneumoniae* was also recovered from kidney, heart, liver, adrenals, and spleen [6] Histopathological examination of lungs of monkeys exposed to NO_2 alone suggested progressive pathology related to the NO_2 concentration The primary effect was reflected by varying degree of expansion of alveoli and by the incidence of septal breaks in the alveoli Monkeys challenged with the infectious agent alone showed areas of alveolar collapse, interstitial lymphocytic infiltration, and fluid edema with only little indication of septal breaks or expansion of alveoli Combined treatment appeared to superimpose rather than potentiate the individual effects of NO_2 and the infectious agents

Pulmonary function measurements in squirrel monkeys reflected changes attributable more to the effects of NO_2 than to the infectious challenge [6] An increased tidal volume with little or no change in the respiratory rate was observed at 10 and 15 ppm of NO_2 Exposure to 35 or 50 ppm resulted in a sharp decrease in tidal volume and a marked increase in respiratory rate The respiratory functions returned to normal within 48 to 72 hr

Effect of chronic exposure Figure 4 illustrates the excess mortalities observed in Swiss albino mice exposed for various lengths of time to 0 5 and 1 5 ppm of NO_2 before respiratory challenge with airborne *K pneumoniae* The increase in death rates after a continuous exposure to 0 5 ppm NO_2 for 3 months or longer was statistically significant Excess mortalities over those in controls were observed in all mice continuously exposed to 0 5 ppm of NO_2 for the shorter time periods, but this was not statistically significant Intermittent exposures to 0 5 ppm of NO_2 for 6 to 18 hr per day resulted in significantly increased death rates after 6 months of exposure [2] However, no increase was observed in those

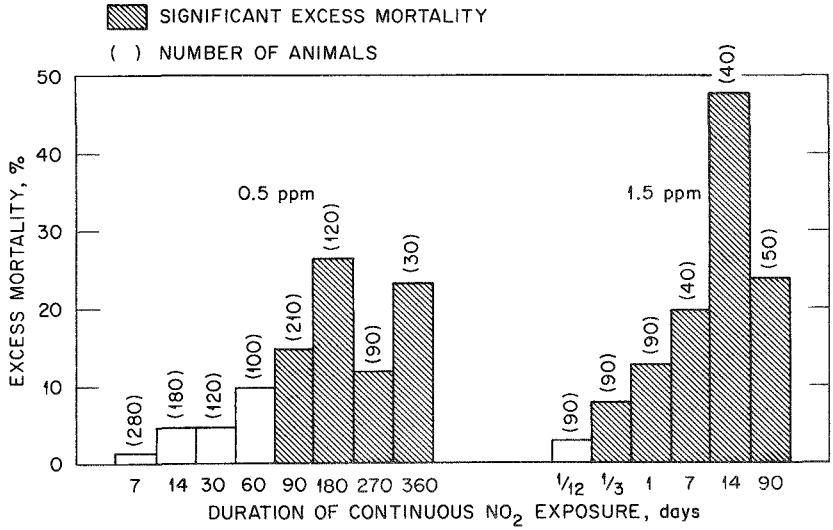

Fig. 4 – Excess mortalities in Swiss albino mice after chronic exposure to 0.5 ppm and 1.5 ppm NO_2 and challenge with *K. pneumoniae.*

two groups after 12 months of exposure. The absence of increased mortality after the 12-month exposure can be explained, at least in part, by the small number of mice available for the infectious challenge.

Continuous exposure of Swiss albino mice to 1.5 ppm of NO_2 resulted in significant excess mortalities after only 8 hr of exposure (Fig. 4).

Histopathological examinations of lung tissues of control untreated mice disclosed the varying degree of pneumonitis that has been routinely observed in most laboratories. In experimental mice exposed to 0.5 ppm of NO_2, we observed inflammation of the bronchioles, surface erosion of the epithelium, and blockage of the bronchiolar-alveolar junction. The alveoli were expanded in all mice exposed to NO_2 from 3 to 12 months, and the number of expanded alveoli appeared to increase with exposure time. Furthermore, the general impression was of early bronchiolar inflammation with reduction of distal airway size and a concomitant expansion of alveoli. The overall lesions appeared to be consistent with the microscopic development of early focal emphysema.[7]

In mice challenged with *K. pneumoniae* only, leukocytic infiltration, edema, and alveolar destruction were seen. Most mice were in the gray hepatization stage of pneumonia. In mice exposed to NO_2 and challenged with the infectious agent, varying stages of pneumonia, bronchiolar inflammation, and alveolar distension were superimposed on the pulmonary pneumonitis.

In order to further define the effect of chronic exposure to NO_2, the size of alveoli was estimated by microscopic measurements. A marked increase in alveolar area was present – the increase being largely related to alveolar expansion and not to septal breakage.[7]

TABLE 1

Effect of NO_2 on Resistance of Squirrel Monkeys to Influenza A/PR-8 Virus

Parameter	NO_2 concentration (ppm)		
	0	5	10
Mortality	1/14* (Died on 6th day)	1/7 (Died on 5th day)	9/10 (All animals died by 4th day)
Respiratory function	Ranged from normal to decreased tidal volume and increased respiratory rates.	Decreased tidal volume and increased respiratory rate by 3rd day. After 2 weeks, respiratory functions normal.	Survivor showed decreased tidal volume and increased respiratory rate.
Erythrocyte sedimentation rate	Ranged from normal to increased rate.	Increased rate.	Survivor showed increased rate.
Body temperature on 3rd day post-virus challenge	Decrease ranged from none to 1.5°F on 3rd day. Moribund monkey had 6°F drop on 6th day.	Survivors: 2.5° to 3°F drop. Moribund animal: 6°F drop.	Survivor showed 3°F drop.
Antigenic response	One week: little or no antibody produced. Two weeks: low to moderate HI and SN. Primary antigenic response (1:12 to 1:96). Excellent secondary response with titers up to 1:2816.	Primary response similar to controls. Secondary response not studied.	Primary response similar to controls. After exposure to NO_2 for 22 days, secondary antigenic response similar to controls.

*No. dead/no. infected.

In further studies of the chronic effects of NO_2 on resistance to bacterial pneumonia, squirrel monkeys were exposed to 5 and 10 ppm of NO_2 for 2 and 1 months, respectively, and within 1 hr challenged intratracheally with *K. pneumoniae.*[8] After challenge, the monkeys were maintained outside the chambers None of the nine squirrel monkeys used as infected controls died. However, two of seven monkeys (28%) exposed to 5 ppm of NO_2 for 2 months died, and one of four (25%) exposed to 10 ppm for 1 month died.

During the 2-month exposure to 5 ppm of NO_2, the tidal volume of the monkeys gradually decreased This decrease was compensated by an increase in the respiratory rates After the infectious challenge the minute volumes of monkeys exposed to NO_2 decreased and remained low, while those of monkeys infected only were elevated This increase could represent a response to overcoming the effect of the infection – the monkeys exposed to NO_2 were apparently not capable of responding in the same manner

During exposures to 10 ppm of NO_2 for 1 month an increase in minute volume was observed, from increases in both the tidal volume and the respiratory rate Within 3 days after the infectious challenge the minute volume increased sharply in controls but decreased in animals exposed to NO_2.

Influenza

Animals were challenged with influenza virus intratracheally 24 hr before exposure to NO_2. This procedure was based on previous studies with synergistic effects of exposure to tobacco smoke and influenza virus[4] and on limited exploratory experiments with squirrel monkeys The results of previous studies indicated that approximately 24 to 48 hr was required for the viral infection to be established before the effects of secondary stress become apparent.

Table 1 summarizes the effects of NO_2 on resistance of squirrel monkeys to influenza infection. In infected control monkeys, 1 of 14 (7%) succumbed to the disease on the 6th day. However, 1 of 7 (14%) and 9 of 10 (90%) died in the groups exposed to 5 and 10 ppm of NO_2, respectively. In the group exposed to 5 ppm the death occurred on the 5th day after the challenge, while in the 10 ppm group all monkeys succumbed within 4 days after the infectious challenge

The surviving monkey in the 10 ppm NO_2 group showed (a) decreased tidal volume, (b) increased respiratory rate, (c) increased erythrocyte sedimentation rate (ESR), and (d) a 3°F drop in body temperature. The surviving monkeys in the 5 ppm group were exposed to NO_2 for 37 days after the infectious challenge. In general there was a decrease in tidal volume and increase in respiratory rate by the 3rd day, but the respiratory functions returned to normal after 2 weeks. An increase in ESR and a 2.5°F to 3°F decrease in body temperature was also observed.

The antigenic response in squirrel monkeys given multiple intratracheal challenges of the influenza virus and exposed either to 10 ppm of NO_2 or to filtered air were observed. No differences were noted in the hemagglutination-

inhibition (HI) or serum neutralization (SN) antibody titers of the experimental and control animals. Both groups exhibited excellent anamnestic response after second injection of the influenza virus.

Retention of Microorganisms in Lungs

Decrease in the ability of an organism to clear inhaled microorganisms from the lungs can serve as an indicator of damage produced by exposure to a gaseous pollutant. To study the effect of NO_2 on clearance of viable *K. pneumoniae,* mice and hamsters exposed to various concentrations of NO_2 were challenged by the respiratory route with the agent. Groups of animals were killed immediately after the challenge and at hourly intervals thereafter. The lungs were removed aseptically, homogenized, and cultured quantitatively. Infected animals not exposed to NO_2 served as controls. The bacterial population of the lungs immediately after the respiratory challenge was considered as unity (100%).

In control mice the viable bacteria population was markedly reduced during the 6 hr after the challenge. Thereafter, the population increased rapidly and reached the initial concentration after about 8 hr. In mice exposed to 5 ppm NO_2 for 2 hr a decrease in viable bacteria was observed during 4 hr after the challenge and the 100% concentration was reached within less than 6 hr. In mice exposed to 25 and 50 ppm of NO_2 for 2 hr the bacterial population decreased during the first hour, but the initial concentration was reached again within 4 and 3 hr, respectively.[5]

Similar response was observed in hamsters exposed for 2 hr to NO_2. In unexposed hamsters, the number of viable bacteria gradually decreased during the first 5 hr, and the initial concentration was reached after 7.5 hr. In hamsters exposed to 5, 35, and 50 ppm of NO_2 the 100% concentration was observed after 6.5, 4, and 4.2 hr, respectively.[5]

Clearance of viable *K. pneumoniae* from lungs of control mice in the chronic exposure studies (1 through 12 months) is shown in Fig. 5. Since the decrease of viable bacteria was approximately linear over a 3-hr period after the infectious challenge, a t_{50} value was calculated. This value expressed the time within which a 50% reduction of original bacterial population occurred. The estimated t_{50} for control mice ranged from 0.9 to 1.2 hr over the 12-month period, with the exception of a 0.5-hr value at 12 months. Mice exposed to 0.5 ppm of NO_2 for 6 hr, 18 hr, and 24 hr per day showed reduced capacity to clear bacteria from lungs after 12, 9, and 6 months of exposure, respectively. At those times the rate of bacterial clearance approximated an arithmetic (t_{50} 1.8 to 2.6 hr) rather than a logarithmic progression (1.0 hr). Irrespective of duration or frequency of exposure, all groups of mice had about 30% of the original bacterial population in the lungs 4 hr after the challenge, and the population remained at this level for up to 7 hr after the challenge. A significant increase, however, was observed in all groups at 24 hr after the respiratory challenge.

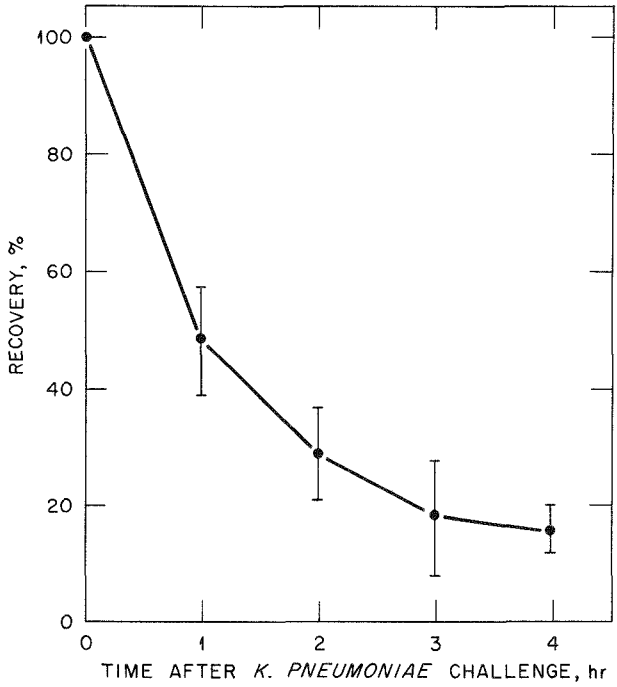

Fig. 5 – Rate of *K. pneumoniae* clearance from lungs of control mice.

Reduced capacity to eliminate bacteria from lungs was also observed in squirrel monkeys exposed to NO_2. Approximately 3 weeks after the infectious challenge, *K. pneumoniae* was present in 1 of 9 control monkeys, 2 of 4 monkeys exposed to 10 ppm of NO_2 for 1 month and 5 of 7 exposed to 5 ppm for 2 months.[8]

COMMENT

Studies summarized in this paper suggest that exposure to NO_2 predisposed animal hosts to experimentally induced respiratory infections by *K. pneumoniae* or influenza virus. Almost irrespective of the duration of exposure or concentration of NO_2, mice, hamsters, and squirrel monkeys showed increased susceptibility to the infections. The altered susceptibility was demonstrated by excess mortality, shortened survival time, and reduced capacity to clear viable microorganisms from lungs.

From the studies of acute effects of NO_2 it is apparent that a 2-hr exposure to the gas almost always resulted in reduced resistance to the infection, and a sharp point of demarcation was apparent in the statistical significance of the differences. Thus for each of the animal species a threshold level could be established below which the enhancement of mortality was not significant, but

above which it was highly significant and apparently not affected by further increases in the concentrations of the gas.

A similar effect was observed in animals exposed to low concentrations of NO_2 for extended periods of time. However, extended exposures to NO_2 of 3 months or longer, as compared to acute 2 hr exposure, reduced the effective dose required to enhance the bacterial infection of the lungs of mice from 3.5 to 0.5 ppm.

In general the responses suggested that alterarions in phagocytic activity in lung may be responsible for the decreased resistance to the bacterial pneumonia. Reduction of activity of alveolar macrophages would permit the inhaled bacteria and viruses to persist in lungs long enough to result in a fatal infection. The pathological changes observed in lung tissues could also contribute to inhibition of surface phagocytosis. In the viral infection, the mechanism involved could also include damage to the interferom-producing mechanisms. The effect of NO_2 on interferon production is presently being investigated in our laboratories.

The phenomenon observed in the studies can be viewed as a highly sensitive indicator of the biological effects of NO_2 in animals. The experimental approach can also be considered as a model system to provide information on the causation of similar diseases in human populations.

ACKNOWLEDGMENTS

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REFERENCES

1. M. R. Purvis and R. Ehrlich, Effects of atmospheric pollutants on susceptibility to respiratory infection. II. Effect of nitrogen dioxide. *J. Infect. Diseases,* 113 (1963) 72–76.
2. R. Ehrlich and M. C. Henry, Chronic toxicity of nitrogen dioxide. I. Effect on resistance to bacterial pneumonia. *Arch. Environ. Health,* 17 (1968) 860–865.
3. M. R. Purvis, S. Miller, and R. Ehrlich, Effects of atmospheric pollutants on susceptibility to respiratory infection. I. Effect of ozone. *J. Infect. Diseases,* 109 (1961) 238–242.
4. A. Spurgash, R. Ehrlich, and R. Petzold, Effect of cigarette smoke on resistance to respiratory infection. *Arch. Environ. Health,* 16 (1968) 358–391.
5. R. Ehrlich, Effect of nitrogen dioxide on resistance to respiratory infection. *Bacteriol. Rev.,* 30 (1966) 604-614.
6. M. C. Henry, R. Ehrlich, and W. H. Blair, Effect of nitrogen dioxide on resistance of squirrel monkeys to *Klebsiella pneumoniae* infection. *Arch. Environ. Health,* 18 (1969) 604–614.

7. W. H. Blair, M. C. Henry, and R. Ehrlich, Chronic toxicity of nitrogen dioxide. II. Effect on histopathology of lung tissue. *Arch. Environ. Health,* 18 (1969) 186–192.
8. M. C. Henry, J. Findlay, J. Spangler, and R. Ehrlich, Effect of continuous exposure to NO_2 on resistance of squirrel monkeys to bacterial and viral infections. *Arch. Environ. Health,* submitted for publication, 1969.

DISCUSSION

D. L. Coffin: Your bar graphs showing NO_2 threshold values for the three respective species [Fig. 3 in text] indicated that the control mortality to *K. pneumoniae* was quite different; that is, you had a rather high control mortality for the mice, and quite a low one for the hamsters and the squirrel monkey. The reverse was true for the NO_2; you needed a large amount of NO_2 for the two latter species, and I wondered whether you would care to comment on the relative role of the bacterium and the gas, in this difference between the species.

R. Ehrlich: Well, we have no real explanation of why it takes more NO_2 to produce the effect in various species of animals. We have tried to correlate several parameters, such as animal weight, the respiratory volume, and so on, in terms of the NO_2 effect, to determine if the difference of about 3 to 30 ppm can be justified; but this simply doesn't work. So it appears that the NO_2 effect is in part species characteristic.

We also know that the hamsters have a high natural resistance to the infection. I don't remember the exact numbers, but we used a considerable number of control hamsters with only very few animals dying. Similarly, the squirrel monkey shows a very high natural resistance to infection.

It's quite possible that by using a different infectious agent to which the hamsters or the squirrel monkeys are more susceptible, we might be able to demonstrate the NO_2 effect at lower concentrations.

We used the *K. pneumoniae* and influenza really as model systems for respiratory infections. We happen to have had considerable experience with these organisms.

R. G. Thomas: You don't seem to be implicating ciliary action at all in this. In other words, you don't seem to think that the NO_2 is effecting the energy systems in the cilia. You seem to be more or less implicating the phagocytic processes. Do you have pretty good evidence for this?

Ehrlich: Basically, I did not want to imply that the ciliary movement and the mucous flow were not affected by the NO_2 exposures. Our evidence does not exclude the cilia, but it strongly suggests that the phagocytic activity might be the more important one in this particular case. This is based on some experiments in which we have infected the animals first with *K. pneumoniae* and then exposed them to NO_2. The increased mortalities persisted for extended periods of time – namely, up to 4 days.

I believe Dr. Coffin in the next paper will discuss in detail some of the mechanisms.

D. Gardner: We have exposed rabbits to various concentrations of NO_2 from 5 to 90 parts per million, and subsequently given them an injection of streptococci in the lung; after a 30-min period we kill the animal, harvest the alveolar macrophages by a lavage technique, and study the *in vivo* phagocytosis rate of these macrophages. We find that at 25 ppm NO_2 there is a 50% depression in the uptake of these streptococci.

M. C. Battigelli: In your continuous exposure experiments, were the control animals kept in parallel conditions? That is, in "sham" exposure chambers?

Ehrlich: Yes, two identical chambers were used.

Battigelli: And my second question is: Could you help me in understanding what is meant by your percent recovery of *K. pneumoniae*? What are your terms of reference?

Ehrlich: Well, the 100% recovery was essentially recovery immediately after the respiratory challenge. In other words, the number of organisms per gram of lung tissue recovered immediately after respiratory challenge was considered to be 100% recovery. The other points represented percent of this 100% recovery.

K. H. Kilburn: Do you have any idea what the comparative alveolar levels of NO_2 are in the three animals? Secondly, do you know whether there is a variable susceptibility of the alveolar macrophages to NO_2 in the three species? Have you cultured macrophages exposed to NO_2 to determine whether squirrel monkeys make more resistant macrophages than mice do? Do you have any other measures of the nitrogen dioxide toxicity in the three animal systems or of the amount of disease that produces fatality in each system?

Ehrlich: You mean different parameters of measures?

Kilburn: Yes.

Ehrlich: Well, there is a considerable amount of pathological, serological, and biochemical work, especially with influenza virus, which is going on at the present time. We have obtained a number of respiratory function measurements and a considerable amount of blood chemistry, enzymological, and pathological data at various time periods after exposure to NO_2 and after infectious challenge. These data have been published in the open literature [see preceding paper for references].

E. Pfitzer: These are extremely interesting and important studies for anyone who is doing work with respiratory irritants. It seems to me that the intriguing question is: What happens when your animal has been exposed to a respiratory irritant and then receives a low-level infection? Do you have any thoughts on

exposure of the animal to low-level infections, and ways to quantitate possible biological effects?

Ehrlich: The study that we conducted with influenza virus and squirrel monkeys was really designed to produce a low-level infection in the animal We hoped to hold on to the animals for extended periods and to look at each individual as a clinical case with respect to total blood chemistry, biochemistry, serology, pathology, etc

Unfortunately, when we infected the animals and placed them in the NO_2 chamber, 9 out of 10 monkeys died, so we were not able to do the low-level infection study

I think in the future we will have to use a different agent system We are considering, for example, using organisms such as mycobacterium to produce a low-level chronic type of infection

STUDY OF THE MECHANISMS OF THE ALTERATION OF SUSCEPTIBILITY TO INFECTION CONFERRED BY OXIDANT AIR POLLUTANTS

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ABSTRACT

Ozone, nitrogen dioxide, and artificial auto smog reduce the ability of animals to cope with aerosols of pathogenic bacteria A model system employing ozone and streptococcus C in mice is being explored to delineate the mechanisms involved Findings to date indicate that ozone elicits a slowing of the so-called bacterial clearance rate in the lung This appears to be the result of a longer maintenance of viability, a shortening of the lag phase, and an acceleration of growth of the introduced organisms — Focal pneumonic areas mainly involving the parenchyma and accompanied by intense accumulations of bacteria frequently unassociated with cellular infiltrate appear to be generally peripheral to alveolar ducts Invasion of the blood by microorganisms begins on the 2nd or 3rd day after exposure to the aerosol, and mortality is probably related more to septicemia than to pneumonia, since large areas of normal lung are usually present even in those individuals with maximal lung bacterial counts It would appear reasonable from the data that ozone exposure not only accelerates growth of bacteria in the lung but promotes invasion of the blood — Experiments performed in rabbits were monitored by the pulmonary lavage method Exposure to ozone elicited a marked influx of polymorphonuclear leukocytes which persisted for more than 24 hr after exposure Concomitantly, there occurred a reduction in the total number of pulmonary macrophages, which built back to normal after 24 hr Associated with this reduction in number was a significant reduction in the ability of these cells to phagocytize bacteria previously introduced into the pulmonary airway Assay of the lysosomal enzymes of macrophages of animals exposed to ozone indicated that lysozyme, beta glucuronidase, and acid phosphatase were markedly reduced Similar reduction also occurred in free lysozymes presumably not derived from the macrophages — The findings reported here indicate that exposure to ozone confers an intensification of the invasion of the lung and blood stream in the mouse streptococcus model If inferences can be made from experiments in rabbits, anatomic, physiologic, and biochemical alterations of pulmonary alveolar macrophages play a major role in this heightened susceptibility to bacterial infection

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Oxidant air pollutants share the propensity of certain other environmental influences to alter the body's ability to cope with infectious agents. Ozone, nitrogen dioxide, and artificial auto smog have been clearly shown to produce enhancement of mortality from infectious aerosols in model systems. The minimal effective doses of these agents required to produce significant mortality differences are: ozone, 0.08 ppm for 3 hr;[1] irradiated auto smog, 0.15 ppm total oxidant for 4 hr,[2] and nitrogen dioxide, 3.5 ppm for 2 hr.[3] Continuing studies in our laboratory are exploring the mechanisms responsible for such enhancement of mortality, as well as the use of the model to obtain information of relevance to air quality criteria by defining minimal effective dose, etc. The mechanism studies are being performed to gain a better understanding of the method by which ozone, apparently the most reactive oxidant gas, reduces resistance to pulmonary infection, and of equal importance, to gain insight into the mode of its toxic action in the lung *per se*. Such information is of immediate relevance to air pollution toxicology since it demonstrates the direct toxic action of oxidants on pulmonary cells. This report summarizes the information to date which is or will be contained in more detailed reports.

After the introduction of living pathogenic bacterial cells into the lungs of normal mice by means of an aerosol, there is a precipitous decline in the number of bacteria which may be cultured from a homogenate of whole lung.

With the *Streptococcus* Group C used in our laboratory, there are normally less than 4 % of the initial deposition of bacteria surviving at 4 hr, and many animals have zero values at 10 hr and beyond. When the mice are pretreated by ozone exposure this decline in the number of cultivable bacterial cells diminishes and is followed by a growth phase in which the organisms eventually become more numerous than at the time of the initial deposition (0 time).[3] A series of experiments were performed to more accurately define the parameters of this bacterial decline and growth, and to relate the changes in bacterial numbers to ozone concentration and to subsequent mortality in the animals. These studies, which are reported elsewhere, will be briefly summarized here. In these experiments the number of bacteria present at 4 hr varied from a mean of 2 % in controls to an actual increase over the zero time value at 1 ppm ozone and above. Median bacterial log values for lungs cultured at 4 days varied from approximately 1×10^2 in controls to $1 \times 10^{3\,2}$ per mouse in the highest ozone concentration (5 ppm), with other levels intergrading sequentially. There was a dose-related slowing of the rate of bacterial decline, a shortening of the lag phase, and an increase in the rate of bacterial multiplication associated with exposure to ozone. The relationship of change in bacterial numbers to both ozone concentration and mortality was highly significant when analyzed by regression analysis.

Invasion of the blood, which began 2 days after exposure, occurred more rapidly in the ozone-treated animals. It appeared to be positively related to mortality in both groups. The post-exposure time at which the median mortality occurred was approximately 4.5 days and 7.0 days, respectively, for the

O_3-exposed and the nonexposed animals. In short, ozone exposure produced increased mortality in the streptococci, with slower bacterial elimination, shortened lag phase, and increased growth of bacteria in the lung. Invasion of the blood was proportional to mortality and appeared sooner in ozone-exposed animals. The earlier death of ozone-exposed animals appeared consistent with the more rapid bacterial growth in the lung and earlier invasion of the blood associated with the ozone treatment.

POSSIBLE MODES OF ACTION IN THE INFECTIVE MODEL

It would seem reasonable to advance the following postulations as possible mechanisms by which oxidants increase mortality to bacterial infection and associated phenomena as described above.

1. Action by nonspecific stress exerted on a systemic level.
2. Reduction of the rate of elimination of infectious organisms by means of reduced mucociliary clearance.
3. The production of anatomic lesions which serve as loci for initial bacterial invasions.
4. Specific action on the pulmonary cellular defense mechanisms (i.e., macrophage system) that produce a lag in bacterial kill.
5. Alteration in the noncellular milieu of the pulmonary airway providing a more salubrious environment for initial bacterial growth.

Experiments performed by introducing streptococci via a nonpulmonary route (intraperitoneal inoculation) after ozone exposure have repeatedly shown no difference in mortality between ozone-exposed and control mice.[3] This would appear to indicate that stress exerted systemically plays no major role in the oxidant effect described in this paper.

A major factor in the oxidant model is the great delay in the speed of removal of cultivable cells from the lungs of mice and other animals conferred by exposure to the gas.[1,2,4] Information has been derived from examination of normal mice by determining the rate of removal of bacterial cells *in toto* by monitoring a radioactive label as compared to the rate of decline in cultivable cells in the same animals. These data indicate that the major portion of the loss of cultivable bacterial cells from the lung is the result of killing *in situ* rather than physical removal.[5] It would thus appear that the vast differences in clearance rate between oxidant-exposed and infected controls cannot be accounted for by delay in the physical removal of bacteria, but only by factors which reduce the lung's ability to render the organisms noncultivable *in situ.*

Exposure to oxidants produces anatomical alteration of the lung. Gross evidence of edema is invariably present after high levels of exposure and death is commonly attributed to this so-called edemagenic effect. However, in the infective model, edema cannot be detected at the lower levels of exposure which

nonetheless produce significant differences in mortality. These low levels are well below those which produce mortality by simple exposure to the gas. Pathological studies of this model are now underway in our laboratory. Preliminary information indicates that ozone exposure results in sporadic focal degenerative lesions in the terminal airways and abutting alveoli. In animals subsequently exposed to bacteria, inflammatory lesions appear to be first related to similar anatomical sites; then they spread diffusely through the lung parenchyma to produce patchy pneumonia and abscesses. When animals are exposed only to the bacteria, however, the early inflammatory lesions appear to be more diffusely spread through pulmonary parenchyma, with frequent association with the larger bronchi. While it may be speculated that the focal ozone lesions exert a predisposing influence for bacterial invasion of the lung in the oxidant mode, conclusions must await further work at lower ozone levels. A detailed study of this aspect of the pathogenesis of the model will be the subject of a later report.

A considerable body of information is developing which indicates that ozone and nitrogen dioxide exposure have a deleterious action on the pulmonary alveolar macrophage, as determined through pulmonary lavage methods.[6,7] Exposure of rabbits for 3 hr to ozone at 0.9 ppm and above produced a significant reduction in numbers and lowered ability of these cells to phagocytize streptococci. These effects appeared transient but persisted for 24 hr.[6] Similar effects were noted for NO_2 exposure at about 10 times the concentration necessary for similar results with ozone.[7] The effect of ozone exposure on three lysosomal enzymes of lavaged macrophages has been determined. Lysozyme, beta glucuronidase, and acid phosphatase were transiently reduced after ozone exposure for 3 hr at concentrations of 0.25 ppm and above.[8] In view of these findings and the known importance of the pulmonary alveolar macrophage in pulmonary defense, it seems reasonable to conclude that this action of the oxidant plays a major role in preparing the lung for the initial persistance and growth of the bacterial invader.

The possibility that the ozone effect may be mediated through some noncellular means cannot be overlooked. It has been shown that ozone exposure reduces the lysosomal enzyme activity in bronchial washings in mice and rabbits, suggesting that the upper airways might present a more favorable site for bacterial residence and eventual multiplication in ozone-exposed animals.[9] While there is no evidence that significant enhancement of mortality in the infective system is associated with edema, methods customarily employed to demonstrate its presence are probably too insensitive to absolutely preclude the possibility that minor edema might be fostering bacterial growth. Finally, no extant data unequivocally rule out the possibility that ozone might have some quantitative or qualitative effect on the noncellular monolayer said to be present in pulmonary alveoli. Further work is required on the reaction of this segment of the pulmonary system to ozone exposure.

From evidence presently available, it would appear that the effect of ozone and other oxidants on the pulmonary macrophage most reasonably account for the altered susceptibility to bacterial infection of the lung conferred by these pollutants. Exactly how this effect is produced and whether other systems are playing a part, either directly by fostering cell persistence and growth or indirectly by mediating the macrophage effect, must await further investigation.

BIOLOGIC IMPLICATIONS OF THE INFECTIVE MODEL

In discussing the importance of the infective system for toxicological appraisal two aspects must be considered. First, there is the possibility that the model may mimic a like situation which occurs or is likely to occur in man. Necessary components for its spontaneous duplication in man are exposure to a sufficient concentration of ozone or other oxidant gas, the presence of an anti-infective mechanism capable of being altered by the exposure, and the presence in the environment of infectious agents capable of exploiting the state of diminished resistance.

Although statistically significant mortality increments have been noted in mice at ozone concentrations well below ambient peaks in many American cities, it is difficult to relate a given effective concentration from mouse to man. Differences in airway length and complexity, velocity of air movement, and the relationship of the volume of air to the area of the target surface in the lungs and other functional and anatomic differences of the respective species would all affect the comparative toxicity of the gas. It would appear that salient information on which to base such an extrapolation could be derived from the construction of appropriate mathematical models. Although sufficient functional and anatomical data are present for man, and probably for the mouse,[10] no discussion of such a model has been noted in the available literature.

Oxidant gases at various concentrations have been shown to enhance the rate of infection in mice,[1-3] rats,[11] hamsters,[3] and squirrel monkeys.[3] It thus seems doubtful that man would be incapable of a similar reaction with like concentrations of the gas delivered to the target site.

Among the infectious agents eliciting this reaction in animals are *Streptococcus pyogenes* Group C, *Klebsiella pneumoniae, Diplococcus penumoniae,* and mouse-adapted influenza A (PR-8). Since the last three agents are human pathogens, our species does not appear to lack this component of the model. In essence the direct application of this model to disease in the human population is still a most crucial point that must be solved by comparative toxicology, and most importantly, epidemiological data.

A second aspect to be considered in the appraisal of this model is its value simply as an exquisitely sensitive indicator of biological effect at an *in vivo* level. The increased susceptibility to infection conferred by oxidant gases is a strikingly clear indicator of biological effect at concentrations probably below that demonstrated by any other objective method. The model therefore has

broader implications than merely showing that susceptibility to infectious disease is increased by oxidants. It indicates that a basic adaptative defensive mechanism of the lung has been impaired. At least a part of this impairment appears as the result of depression of the macrophage system by action of the oxidant. It is generally concluded that the macrophage constitutes one of the most important defensive mechanisms of the deeper lung. It has been postulated, for instance, that transport and storage of carcinogen-containing particles is carried out through the agency of the pulmonary alveolar macrophage. Furthermore, since ozone exposure has been shown to reduce the level of three lysosomal enzymes (beta glucuronidase, acid phosphatase, and lysozyme), there is a possibility that induced anticarcinogenic enzymes within the lung might react similarly. It would thus appear reasonable to investigate these phenomena from the standpoint of their role in carcinogenesis.

REFERENCES

1. D. L. Coffin, E. J. Blommer, D. E. Gardner, and R. Holzman, Effect of air pollution on alteration of susceptibility to pulmonary infection. Third Annual Conference on Atmospheric Contamination in Confined Spaces, Dayton, Ohio, May, 1967.
2. D. L. Coffin and E. J. Blommer, Acute toxicity of irradiated auto exhaust indicated by mortality from streptococcal pneumonia. *Arch. Environ. Health,* 15(1967) 36–38.
3. R. Ehrlich, Effect of nitrogen dioxide on resistance to respiratory infection. *Bacteriol. Rev.,* 30(1966) 604–614.
4. D. L. Coffin and E. J. Blommer, Alteration of the pathogenic role of *Streptococci* Group C in mice conferred by previous exposure to ozone. In, *Proceedings of the Third International Symposium on Aerobiology, University of Sussex, England, September, 1969.*
5. G. M. Green and E. H. Kass, Factors influencing the clearance of bacteria on the lungs. *J. Clin. Invest.,* 43(1964) 769–776.
6. D. L. Coffin, D. E. Gardner, and R. S. Holzman, Influence of ozone on pulmonary cells. *Arch. Environ. Health,* 16(1968) 633–636.
7. D. E. Gardner, R. S. Holzman, and D. L. Coffin, Effects of nitrogen dioxide on pulmonary cell population. *J. Bacteriol.,* 98(1969) 1041–1043.
8. D. J. Hurst, D. E. Gardner, and D. L. Coffin, Effect of ozone on acid hydrolases of the pulmonary alveolar macrophage. *J. Reticuloendothelial Soc.* submitted for publication, 1969.
9. R. S. Holzman, D. E. Gardner, and D. L. Coffin, *In vivo* inactivation of lysozyme by ozone. *J. Bacteriol.,* 96(1968) 1562–1566.
10. Charles R. Key, Morphometry of Mouse Lungs. Ph.D. Thesis, University of Oklahoma, Norman, 1966. Based on work performed in the Experimental Pathology Section, Biological Research Branch, Division of Health Effects Research, National Air Pollution Control Administration, Cincinnati, Ohio.
11. E. J. Blommer, unpublished data.

DISCUSSION

E. P. Radford: Dr. Coffin had us all on the edge of our seats waiting for the answer to the big question: Where do the macrophages come from? Would you want to hazard a guess, Dave?

D. L. Coffin: Well, I can only tell what the other fellows say: I think that, first of all, there are classically two schools of thought concerning the origin of macrophages. One is that they originate in the lung, from cells existing there. These are lung macrophages which are a special variety.

The other school states that the macrophages come in from the blood, and they are differentiated monocytes. Being a coward, I have subscribed to both schools. I feel that about half of the macrophages come from the blood, and half of them are made in the lung. I think that there are some very sophisticated investigators who also subscribe to this view. They debate the respective proportions derived from each source. Some investigators, for instance, claim that 30% come from the blood and 60% from the *de novo* manufacture in the lung. I plainly don't know the exact proportion derived from each source.

However, I mentioned a plateau that we get in dose effect. We've seen this both in the changes in the cells (that is, the effect on numbers of macrophages) and in the effect on the lysosomal enzymes. A plateau occurs at about 3 or 4 ppm of ozone, but this is not a typical dose response. Now, why is this? It could very well be that it is some sort of two-cell population thing, or it could represent an influx from the bloodstream of new cells not affected by the ozone and therefore they appear normal. We don't have the answer to your question, but I think it's a very interesting one nevertheless.

C. Loosli: You're not satisfying that gentleman, David; he's still standing!

Radford: I want to amplify what Dr. Coffin just said because I think that wherever the macrophages come from, it is a highly dynamic system. One way to look at the macrophages is to compare the absolute numbers that you can wash out from the lung with the alveolar surface area, or the number of alveoli. When you do this, for the Greenacres strain of rats we've used, you get about one washable macrophage per three alveoli. If this were not a dynamic system, that is, one with cells moving rapidly out through bronchial clearance, it wouldn't be a very effective system for doing much clearing within the lung. As an explanation of your ozone experiments, the most likely is that the equilibrium of free alveolar cells has been reset at a lower level.

U. Saffiotti: Dr. Coffin, do you find lesions *in situ* in the tissues similar to those you described in the macrophages that were collected from washings? I have somehow always thought of the pulmonary macrophage as a cell active in the tissues, which, at the end of its life cycle, may eventually drop into the air passages.

In relation to this, did you attempt to measure what the percentage of macrophages washing out into the air space is in comparison to those present in the tissues? Is the number of macrophages present in the tissues affected by the experimental exposures you described?

Coffin: Dr. Saffiotti always asks the $50 question! We don't know what these effects are in the macrophages remaining in the lung. We really have no

means of determining them by most of our methods. We have not studied this problem by electron microscopy, but work of this type is in progress now.

I will say that when one lavages the lungs, no matter how long the lavage is continued, there are still a lot of macrophages remaining. We don't claim that we are eliminating the total population of macrophages. On the contrary we are getting some sort of sample. We don't know how biased it is, but we are getting a sample; and our method has been used by a number of other investigators.

R. Rylander: I would like for a minute to draw attention to the presently somewhat neglected mucous ciliary clearance system. In the data Dr. Coffin cited from G. M. Green and E. Kass [*J. Clin. Invest.*, 43 (1964) 769], I think it is important to bear in mind that in these experiments the trachea and a large part of the bronchi were not included in the preparation. It is thus really a question of whether you look upon the lung as the total organ below the larynx, in which case mucous ciliary clearance will be of greater importance, or whether you are interested primarily in the parenchymal portion of the lungs.

The other point I would like to make is that the relative importance of the two systems in inhalation toxicity might be related to the length of the exposure. We use a method whereby – apart from introducing viable bacteria in the lungs of the animals – we simultaneously introduce particles to get a measurement of mechanical clearance in the same animal. We found that the effect on the mechanical clearance usually does not appear until after 2 or 3 weeks of almost continuous exposure when moderate exposure levels are used. Thus, if we study the animals at 4 weeks after exposure to SO_2 and dust, we have a depression of the mucous ciliary clearance. This in turn reduces the mechanical reduction of viable bacteria, which could mean that the phagocytes are not affected. This is why the data that indicate a plateau on the effects of the phagocytes are especially interesting. Do you have any data showing that there is a return to normal after continuous exposure, which could then tie in with our observations?

Coffin: We don't have data on continuous exposure. All the data here are derived from one exposure for 3 hr.

Rylander: As there is sometimes a discrepancy between the interpretation of results from various methods in this field, maybe we should all try to use animals subjected to various standard exposures and apply the various methods available. Perhaps this could be a useful way to further elucidate the relative importance of the mucous ciliary system and the phagocytic function in inhalation toxicity.

Coffin: That's a very interesting idea. As I say, we have not done any chronic exposures; they have all been acute. I will say that at the 4-hr period at the lower levels of ozone, there is no difference in the rate of clearance from the normal. I think it's a little difficult to postulate that there would be an alteration of

physical clearance at this level, because it is the same as the normal level. Although, subsequently, lung infection seems to occur at this point, it would appear that the difference in the rate of infectivity is apparently built from a rather similar base of organisms. This is why we are postulating that there is a great importance in this antibacterial defense mechanism in which bacteria are destroyed *in situ.* This defense is depressed by ozone exposure, thereby permitting the organism present to reproduce and produce disease.

G. Freeman: I thought I might help complicate the picture a bit by adding a third variable and by showing some pictures, if I may, of alveolar structures from rats that have been exposed to a subacute level of NO_2 (17 ppm) for 4 to 8 hr. We are currently down to 2 ppm, but material is not yet ready to show at this level.

The slides were prepared by Dr. Stephens of our laboratories, and point out several interesting effects at the alveolar level. You see in the first slide [Discussion fig. 1a], in the upper left-hand corner, blebbing of the epithelium, which is abnormal for the alveolar septum. This was taken at 4 hr. There is considerable distortion of the structure of the epithelial cell on top and, below, you can see strands of fibrin accumulating in the alveoli.

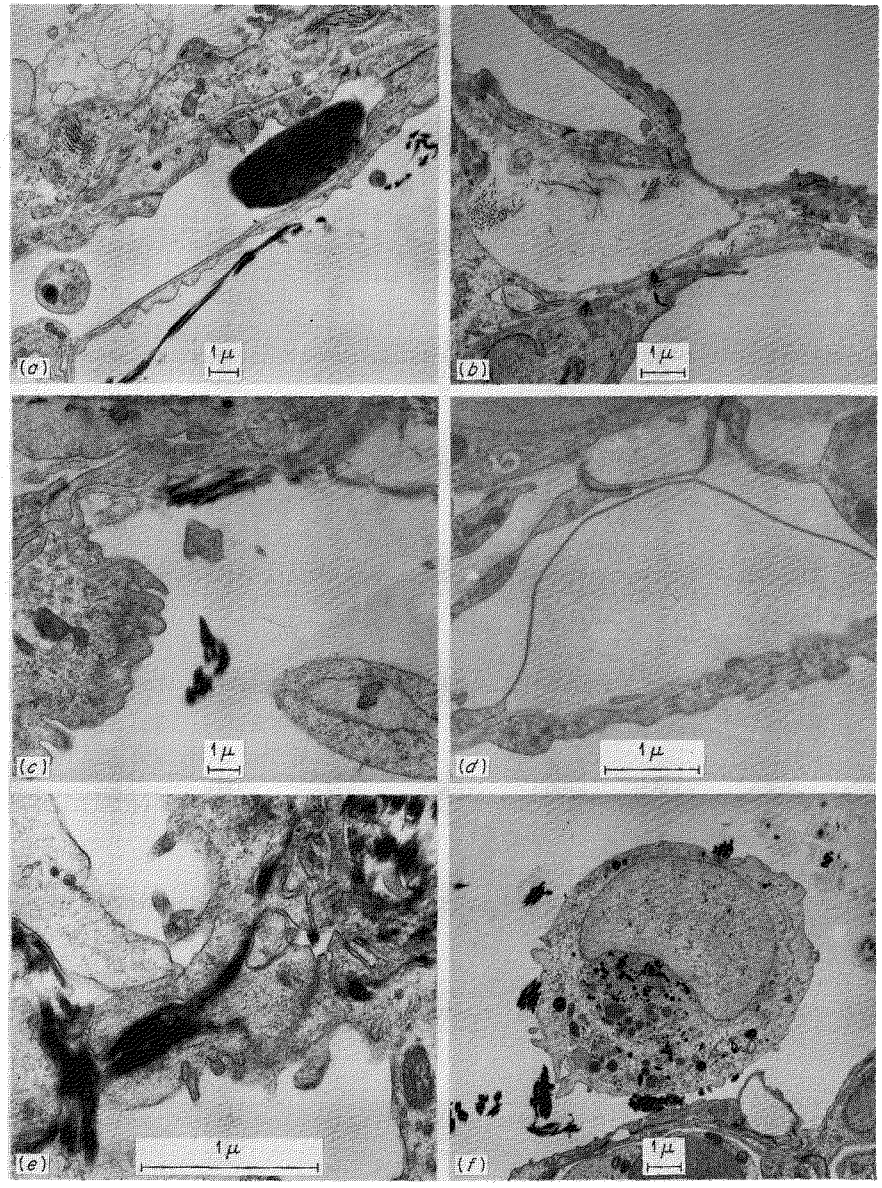
The next slide [Discussion fig. 1b] shows another type of change. The "pool" in the left center is in the basement membrane. There is a tendency for the membrane to "dissolve" focally, one might say, and form small lakes. You can see collagen in the center.

In slide 3 [Discussion fig. 1c], in the center at the top, is basement membrane, below which the epithelial cell is "chewed up," or absent. What is sitting on and within the basement membrane, because it is exposed, is more fibrin. This slide shows a destructive lesion of the epithelial surface itself, which is not seen with the light microscope.

There is another kind of lesion here [see Discussion fig. 1d], an endothelial one. Notice that tent-like structure; it is an endothelial cell which has become very attenuated on the left side, and then has stretched out to form a sort of a blister in the lumen of the vessel. This is seen quite readily and is, apparently, an endothelial lesion caused by the NO_2.

On the right half [of Discussion fig. 1e] is a macrophage that is engulfing fibrin. You can see the fibrin in the cell and between the macrophage and the epithelial cell that it is approaching. The extravascular fibrin implies vascular porosity.

In the last slide [Discussion fig. 1f] is seen fibrin around a macrophage in the alveolus. I'm showing these as fairly clear evidence of injury at the alveolar level, which may allow bacteria or other injurious materials, such as carcinogens, to become incorporated into the cells and possibly to be carried into the circulation.



Discussion fig. 1 – Alveolar structures from rats exposed to a subacute level of NO_2 (17 ppm) for 4 to 8 hr. (a) Blebbing of the alveolar septum of Type 1 cells, 4 hr; (b) pool in basement membrane, with collagen in center; (c) lysis of Type 1 cell showing deposition of fibrin within and on the basement membrane; (d) separation of endothelial cell from septum; (e) phagocytosis of alveolar fibrin; (f) fibrin in alveolus.

Loosli: Would you like to comment on these slides?

Coffin: I think they're very beautiful photographs; I wish I had taken them! With the light microscope we do see changes from exposures to ozone at 2 ppm and above, and since these changes could conceivably be the same sort of lesion that Dr. Freeman has shown here, I would like to reiterate the possibility that there could be an anatomical component in this thing.

P. Nettesheim: I would like to make a comment. Dr. Coffin made a very shy attempt to relate his findings to carcinogenesis, suggesting that NO_2 exposure, or ozone exposure, might also have some effect on the clearance of carcinogenic substances. Since this is an inhalation carcinogenesis conference, I would like to stress this point in a somewhat different manner. I think there is, from different laboratories, more and more evidence that the microbiological status of the animals may be important not only in terms of the number of tumors that develop after carcinogenic treatment, but also in terms of the type of tumors that develop. I hope to be able to give some suggestive evidence tomorrow in this regard.

If this suggestion holds true, then I would say that any means which lowers the resistance of the animal to cope with infectious agents in the lung could be of extreme importance in terms of inhalation carcinogenesis.

EFFECTS OF NO_2 IN HAMSTERS: AUTORADIOGRAPHIC AND ELECTRON MICROSCOPIC ASPECTS

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ABSTRACT

Nitrogen dioxide (NO_2) has been used as an agent to study experimental injury and repair in the lungs of hamsters. Although repair is nearly complete within 2 weeks after acute exposure, a transitory, localized, and delayed hyperplasia is observed in the region of the respiratory bronchiole. The dynamics of this epithelial replacement has been studied by tritiated thymidine labeling and autoradiography. After an acute 6-hr exposure to 100 ppm of NO_2, there is an intense burst of epithelial proliferation which peaks at about 24 hr. This peak is most intense in the major bronchi, returning to normal levels within 4 days. In the distal respiratory tract, the peak is delayed until 48 hr, is lesser in degree, and returns to normal levels within 7 days. This increase in thymidine index persists to an even greater extent in the respiratory bronchiolar region after prolonged NO_2 exposures. — Electron microscopic study of NO_2-injured hamster lungs has revealed the following. The ciliated cells do not change in number, but the number of secretory cells and secretory granules or inclusions diminishes precipitously. Lysosomal structures increase dramatically after NO_2 exposure. In the distal lung, the number of type II alveolar cells increases in the respiratory bronchiolar and alveolar regions, as does the number of phagocytic cells. These findings suggest a depression of mucopolysaccharide production, an increase in surfactant or lung phospholipid production, and an increase in lysosomal enzyme content.

For the past few years we have been interested in the events surrounding injury and repair of the lung and tracheobronchial tree. Our test agent has been nitrogen dioxide (NO_2) administered by inhalation techniques for periods of time varying from several hours to several months and in concentrations varying from 20–30 ppm in some studies to 40–50 ppm in others. Our interest in the effects produced by these kinds of exposures derived from a primary interest in producing a model of human emphysema. It was immediately apparent in our early studies that inhalation of sublethal concentrations of NO_2 for periods of over several hours produced cell necrosis in the tracheobronchial tree, acute

pulmonary edema in the alveolar regions, and a proliferative response that was particularly prominent in the region of the terminal and respiratory bronchioles 72–96 hr after the acute exposure. We reported[1] several years ago that even after 10 weeks of continuous exposure to NO_2 in concentrations of 50 ppm, the lung volumes that increased immediately after exposure reverted toward normal values after 2 weeks of recovery in air. The histological studies did not reveal convincing evidence of tissue destruction, and we concluded that anatomic emphysema had not been produced. There was, however, no escape from the observation that intense cellular reactions had occurred, even if destruction had not, and we became absorbed in the study of these cellular reactions. One of the first problems that presented itself concerned the reactivity of the various portions of the respiratory tract. Our histologic studies had indicated a proliferative response in the respiratory bronchiolar areas 3–4 days after an acute exposure to moderate NO_2 levels. This response persisted even after several months of continuous exposure but appeared less striking and less intense. The trachea and major bronchi never appeared to have the intensity of proliferative activity seen in the respiratory bronchiolar regions, and the alveoli also failed to show this dramatic epithelial response and were not edematous following chronic exposures. These observations suggested a variability of responses at different sites in the respiratory tract in spite of experimental designs which provided for continuous, steady-state exposures of NO_2 that would subject all parts of the respiratory tract to relatively equal concentrations. We studied this problem by labeling cells with tritiated thymidine. This technique consists of labeling the DNA of the nucleus during the premitotic synthetic "S" phase of the cell cycle and identifying the labeled cells by subsequent autoradiography. It was necessary, first, to establish values for normal animals.[2] Our test animals were male Syrian golden hamsters weighing from 80–100 g. It was our feeling that calculation of the thymidine index would give sufficient information to evaluate the relative activities in various portions of the respiratory tract. Each animal was given a series of four intraperitoneal injections of *methyl*-^{3}H-thymidine (^{3}HT; specific activity 6.7 Ci/mmole) at hourly intervals beginning at 9:00 a.m.; each injection contained 0.5 μCi/g body weight. Multiple injections were used to increase the proportion of labeled cells and thereby the accuracy of counts. Animals were sacrificed by rapid exsanguination 2½ hr after the last injection, and complete autopsies performed. Lungs were fixed in the expanded state by warm formalin vapor at 55°C and 20 cm of water-positive pressure for 24 hr, while floating in 10% neutral-buffered formalin. After post-fixation in Bouin's, sections were taken from the lung and trachea of each animal. Three sections of 5-μ thickness were cut from each block; one was prepared for H & E section and the others were processed and dipped in NTB3 liquid emulsion. These slides were exposed for 3 weeks at 4°C, developed in D-19, stained, and counted. Thus, four slides, each with a cross section of trachea and a section of lung along the plane of the main bronchus, were prepared for each animal. Thymidine indices (number of labeled

nuclei per thousand) were determined on five epithelial sites in the airways: (a) trachea; (b) main bronchus; (c) medium-sized bronchi (interlobar); (d) preterminal, terminal, and respiratory bronchioles; and (e) alveoli. Alveolar studies were performed in only nine animals, with two populations of alveolar cells determined; these were designated "septal" and "non-septal." Alveolar macrophages and other cell elements were not counted. Five hundred nuclei were counted at each level on each slide for a total of 2000 nuclei at each level per hamster. A nucleus was considered labeled if there were more than five silver grains overlying it.

The thymidine indices of the various cell populations studied are shown in Table 1. The trachea, with an index of 6.4 per 1000, is distinct from other conducting airways. There is relatively uniform labeling from main bronchus to respiratory bronchiole, with no significant differences between any of the intrapulmonary conducting airway cell populations. The two alveolar cell populations are clearly distinct. The septal cells have an index of 18.3/1000 and the non-septal cells one of 4.9/1000. This is a statistically significant difference. The results observed in the trachea are similar to those observed by Shorter *et al.*[3] and by Blenkinsopp[4] in the rat. With regard to the alveolar cells, it has been reported by Bertalanffy and Leblond[5] and Shorter *et al.*[6] that two distinct populations of cells exist, one with a renewal time 3 to 5 times that of the other.

TABLE 1

*Thymidine Indices in Hamster Lung**

Cell population	Number of hamsters	No. labeled nuclei per 1000 (mean ± S.E.)
Tracheal epithelium	28	6.40 ± 0.87
Main bronchus epithelium	28	2.55 ± 0.41
Medium bronchus epithelium	28	2.32 ± 0.48
Preterminal, terminal, and respiratory bronchioles	28	2.64 ± 0.49
Alveolar "septal" cells	9	18.3 ± 0.1
Alveolar "non-septal" cells	9	4.9 ± 0.9

*After 0.5 μCi/g ^{3}HT once an hr for 4 hr.

Similar studies were performed with hamsters exposed to 100 ppm NO_2 for $5\frac{1}{2}$ hr. The methodology was essentially similar to that described for the control studies. Hamsters are exposed in a 30″ × 24″ × 24″ lucite chamber with a tapered mixing chamber at the top. NO_2 is introduced from a tank of pure NO_2, controlled by a flow meter and needle valve, and mixed with unfiltered room air. Effluent is exhausted through a series of charcoal filters to a hood. The chamber

is under 2-cm H_2O negative pressure, and airflow is maintained at 200 liters/m, enough to exchange the chamber volume every 1½ min. NO_2 concentration is measured continuously by a mast meter and recorded on a 2.5 mv Honeywell-Brown recorder. The mast meter is calibrated two times daily against chemical analyses for NO_2 performed by the method of Saltzman. Temperature and humidity are recorded continuously. After thymidine injection as described for controls, all animals are sacrificed 2½ hr after the last ^{3}HT injection. Sacrifices and labeling are performed at 6 hr and at 1, 2, 4, 7, and 14 days after initiation of the NO_2 exposures. In exposed animals 10,000 nuclei are counted at each of the levels of the conducting airways noted previously and in the alveoli, in each of the sacrifice groups.

As shown in Fig. 1, the three upper levels of lung (trachea, main bronchi, medium bronchi) responded in similar fashion in the NO_2-exposed animals. At 6 hr after the initiation of exposure the thymidine index (TI) in all sites was lower than in comparable sites in controls, but this is not a statistically significant difference. At 24 hr the mean tracheal TI was 12 times the control index, but soon returned toward normal and at 48 hr was only three times the control value. The main bronchi showed an enormous TI at 24 hr, totaling 49 times the control value. This tremendous relative increase is in part due to the lowered resting TI of the main bronchus as compared to the trachea, but is also due to the fact that the cells at this level have the highest absolute labeling index. At 48 hr the TI of the main bronchi falls to eight times the control value and is normal at 4 days. The medium (interlobar) bronchi at 24 hr after injury have a TI that is 36 times the control index. At 48 hr it is only eight times the control value and by 4 days has returned to normal. At 24 hr the region of the preterminal, terminal, and respiratory bronchioles reaches a TI that is 23 times the control value. It falls to 19 times the control index in 48 hr, is still two times the control value at 4 days, but returns to normal in 7 days (Fig. 1).

In the alveolar population, those cells categorized by us as "septal" respond to the severe NO_2 injury by showing a TI that is only two times the control value at 24 hr, but this index increases to three times the control value at 48 hr, before beginning its descent to normal levels. However, even after 7 days post-injury the TI of septal cells is 1½ times the control value, and this is a significant difference. The non-septal cells show a similar course and magnitude of changes but have returned to control levels by 7 days post-injury.

These studies emphasize the individuality of response in the various parts of the tracheobronchial tree after acute injury and the rapidity with which the proliferative activity disappears and returns to normal levels. The greatest intensity of response is noted in the main bronchi, but the reason for this area having the strongest proliferative faculties is not apparent. The findings in the preterminal, terminal, and respiratory bronchiolar region are consistent with those observed histologically; that is, there is a residuum of cell proliferation in this locus continuing through the 4th day without evidence of similar activity in other loci of the tracheobronchial tree. Thus, exclusive of the trachea, there

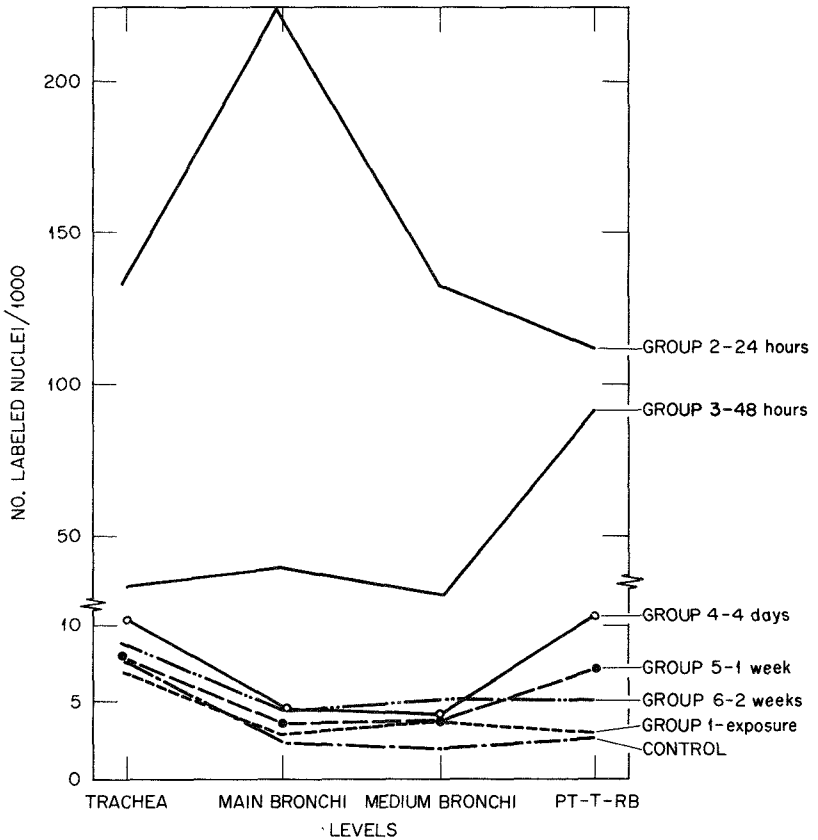


Fig. 1 – Thymidine indices in various parts of the hamster respiratory tract for each period of time after NO_2 exposure. Note the different scales on the ordinate. Alveolar labeling levels have not been included on the graph. PT-T-RB = preterminal, terminal, and respiratory bronchioles.

appears to be a gradient of decreasing proliferative response from main bronchus to alveolus, with an inverse gradient from alveolus to main bronchus as regards the disappearance of the proliferative activity. Stated in another way, the most intense cellular proliferation occurs in the main bronchus and decreases in intensity as the alveolus is approached, while the proliferative activity persists longest in the alveolus (septal-type cells) and exists for shorter periods as the bronchial tree is ascended. We would prefer to think that these differences are the result of intrinsic variations in metabolic and renewal mechanisms of the different cells in the various loci of the tracheobronchial tree.

These differences in proliferative response persist and can be observed in chronic as well as acute injury. Similar studies with smaller groups of hamsters exposed for 8 days at 50 ppm, and for 64–66 days at 20 ppm, demonstrate a

significantly increased TI in the preterminal, terminal, and respiratory bronchiolar region.

This individuality of proliferative response to NO_2 injury suggested to us that a more detailed study of the cellular characteristics of the bronchioles and alveoli might increase our insight and our understanding of the cellular responses in these regions. We therefore initiated studies of thin (1 μ), epon-embedded sections using light microscopy and electron microscopy. Both control and NO_2-exposed animals were prepared at sacrifice for study by these methods. Exposed animals had received (a) 20 days of 40 ppm NO_2 or (b) 10 weeks of 50 ppm NO_2. Tissue was fixed in buffered gluteraldehyde and post-osmicated in cacodylate buffer at pH 7.2–7.4. Sections were stained with 2% uranyl acetate for 45 min and 1.5% lead for 15 min. One-micron epon sections were stained with toluidine blue (TB), periodic acid–Schiff (PAS), or PAS-TB. Attention was focused on three distinct anatomical loci: the intrapulmonary bronchioles, the respiratory bronchioles, and alveoli.

In the normal bronchiole two main cell types can be observed – the classic ciliated cell and the secretory cell. The ciliated cell is cuboidal and contains only a rare PAS-positive cytoplasmic inclusion, which we believe to be lysosomal in nature. The secretory cells are characterized by the presence of blebs of varying size and number. These structures are cytoplasmic protrusions that may be attached to the lumenal cell surface or lie free within the lumen. Within the blebs or main body of the secretory cells are cytoplasmic inclusions which stain homogeneously with toluidine blue. When viewed with the electron microscope the bleb-like structures are composed of masses of smooth endoplasmic reticulum, characteristic of many secretory or endocrinal cells. In addition, when examined by light microscopy, the granules characterized by their affinity for toluidine blue are round or oval bodies, homogeneous in character, single-membrane bound, and not particularly electron dense. Other cytoplasmic elements, such as ribosomes, mitochondria, Golgi, and at times even a nucleus, are also present in these structures.

PAS granules are found more frequently in the ciliated cells in control animals. These PAS-positive structures, which are about 0.2 μ in diameter, are generally larger than the toluidine blue granules and are frequently present in the supranuclear region. The toluidine blue granules are often found in clusters of 10–20, are about 0.1 μ in diameter, and are found only in secretory cells. After exposure to NO_2 it can be seen that there is a decrease in both the total number of toluidine blue inclusions and the number of cells containing these inclusions. In addition, the number of cells with blebs decreases markedly after NO_2 exposure, whereas the number of ciliated cells does not change (Table 2A). The PAS-positive inclusions increase markedly in total number, in the number of PAS inclusions per cell, and in the number of cells containing PAS inclusions after exposure to NO_2 (Table 2B). In the respiratory bronchioles the number of type II alveolar cells shows a moderate increase, while the total number of vacuoles within the type II cells increases markedly. A much less impressive

TABLE 2

Bronchiolar and Bronchial Parameters in Hamster Lung

A. Bronchiolar epithelium

Treatment	No. animals	Total nuclei	Cell height (μ)	Cells with TB inclusions	TB inclusions	Cells with blebs	Ciliated cells
				(all per 100 nuclei)			
Controls	3	1906	0.69	31	159	28	50
NO_2 exposure	4	2797	1.76	9.5	102	1.5	58

B. Bronchial epithelium

Treatment	No. animals	Total nuclei	Cells with PAS inclusions	PAS inclusions	PAS inclusions per cell
			(per 100 nuclei)		
Controls	3	957	10	13	1.4
NO_2 exposure	4	2446	40	220	4.4

Abbreviations: TB, toluidine blue; PAS, periodic acid–Schiff.

increase is noted within the phagocytic type of free or detached alveolar cells (macrophages). The total number of red blood cells present also shows a decrease, suggesting a decreased perfusion within the lung (Table 3A). Similar changes are observed in the alveolar epithelium (Table 3B). These observations suggest strongly that secretory activity is inhibited while structures and materials needed in the processes of phagocytosis and scavengerization are much increased.

One unique and interesting response is observed in the region of the respiratory bronchiole. The cellular response after the prolonged exposure appears to be predominantly in an elongated cell related to the type II alveolar cell. It is characterized, as is the type II cell, by the presence of microvilli on the surface and laminated inclusion bodies similar to those seen in the alveolar type II cells. Frequently, these cells surround and grow over involuted or "washed-out" cells which appear to be nonviable. This mechanism is suggestive of that originally described by Hulse[7] in the respiratory bronchioles of animals with lung injury. The nature and purpose of this response is yet to be ascertained.

These morphological observations are suggestive of certain biochemical alterations that may be occurring in the epithelial cells of the lung. The decrease in toluidine blue granules in the secretory cells points to a depression of mucopolysaccharide production. The increase in lysosomal structures in the bronchiolar cells, as well as the increase in laminated bodies in the epithelium covering the respiratory bronchioles, suggests an increased production of

TABLE 3

Respiratory Bronchiolar and Alveolar Parameters in Hamster Lung

A. Respiratory bronchiolar epithelium

Treatment	No. animals	Total nuclei	ALV II cells	Total VAC in ALV II cells	Phag. cells	Total VAC in phag. cells	RBC
Controls	3	500	15	210	3	59	146
NO_2 exposure	4	873	27	715	11	79	94

B. Alveolar epithelium

Treatment	No. animals	Total nuclei	ALV II cells	Total VAC in ALV II cells	Phag. cells	RBC
			(all per 100 nuclei)			
Controls	3	1100	16	238	4	132
NO_2 exposure	4	1300	22	401	8	77

Abbreviations: ALV II cells, type II alveolar cells; phag., phagocytic; VAC, vacuoles.

enzymic and perhaps lipid substances known to be associated with these structures.

The complexities involved in the response to injury by NO_2 are many, and our observations have created more problems and challenges than answers. The unique response of the respiratory bronchiolar regions to injury has been documented. The alterations within the cells of the bronchiolar regions, including the decrease in cell blebbing and the decrease in toluidine blue inclusions of the secretory cells, require further study for understanding. Why should secretory function be reduced, if indeed it is? The increase in lysosomal structures in the ciliary and secretory cells suggests an increased proteolytic activity. Is their function more than scavengerization and mop-up? The purpose of the notable increase in the type II alveolar-like cells in the respiratory bronchiole and alveoli is not understood. If there is increased production and secretion of phospholipid in these areas, what is its purpose? It is obvious that there exists no lack of problems, and faced with these many challenges the path of the experimental pulmonary pathologist is straightforward, if not easy. Perhaps an understanding of the cellular responses to this relatively simple insult may help in unraveling the much greater complexities involved in neoplasia. In any event, let us get on with it.

ACKNOWLEDGEMENTS

The work presented herein is the fruit of the combined efforts of the staff, both technical and professional, of the Department of Pathology Research, Saint Luke's Hospital. To them, my humble thanks.

REFERENCES

1. J. Kleinerman and C. R. Cowdrey, The effects of continuous high level nitrogen dioxide on hamsters. *Yale J. Biol. Med.*, 40 (1968) 579.
2. C. R. Cowdrey and J. Kleinerman, Tritiated thymidine index and turnover time at various levels of the respiratory epithelium of the hamster. *Federation Proc.*, 27 (1968) 250.
3. R. G. Shorter *et al.*, Cytodynamics in the respiratory tract of the rat. *Thorax*, 21 (1966) 32.
4. W. K. Blenkinsopp, Proliferation of respiratory tract epithelium in the rat. *Exptl. Cell Res.*, 46 (1967) 144.
5. F. D. Bertalanffy and C. P. Leblond, The continuous renewal of two types of alveolar cells in the lungs of the rat. *Anat. Record*, 115 (1953) 515.
6. R. G. Shorter *et al.*, Cell turnover in the respiratory tract. *Dis. Chest*, 46 (1964) 138.
7. E. V. Hulse, A concept of dust disposal in the lungs. *J. Pathol. Bacteriol.*, 69 (1955) 225.

DISCUSSION

G. Buell: I'm curious as to why you people use such high concentrations of NO_2, which are several times what you would find in the atmosphere. There must be a reason for it.

J. Kleinerman: The reason is that I'm an experimental pathologist, and I'm interested in the responses of tissue to injury.

Buell: Injury in general, and not just injury from NO_2?

Kleinerman: To injury. NO_2 is my tool.

H. G. Boren: I have two questions. How many animals were killed at each time? I ask this because I think that sufficient animals should be killed at each time interval to avoid the effects of unknown factors, such as intercurrent infection.

Kleinerman: Six to eight animals at each time.

Boren: The second question: What was the time sequence of the ultrastructural changes in relation to the autoradiographic labeling? Did the ultrastructural changes precede the increased labeling, or not?

Kleinerman: These were two distinct studies.

Boren: But do you know the time relationships? Do you know whether or not the EM changes preceded the increased labeling?

Kleinerman: Well, the labeling experiment was done primarily with 100 ppm and was a completely different experiment. The EM studies were done on animals chronically exposed for either 20 days or 10 weeks.

Boren: The reason I ask this is perfectly obvious: Are the ultrastructural observations simply preparations for mitosis, which could be detected more effectively by autoradiography?

R. T. Drew: I would just like to point out that the literature is full of conflicting reports regarding the toxicity of nitrogen dioxide, and I would like to suggest that all investigators take extreme care with gas-feed systems to be sure that no impurities other than NO_2 enter the system. Care should also be taken with the analytical procedures. Finally, just to further confuse the picture, I've exposed hamsters to what I think is a nearly identical situation, 100 ppm of NO_2 for 6 hr, and either they don't even survive the exposure or, if they do, they die within 3 hr later.

Kleinerman: Of course, we killed the first group of our animals 2½ hr after exposure, but all of our animals survived the exposure. I pointed out to you the intensity of our analyses: they were calibrated twice daily at the beginning, and the chamber was turned on for at least an hour before the animals were put in. Multiple samples were taken before we decided that we had reached the proper level.

But it should be recalled that we had to open the chamber for 5 min out of each hour in order to inject the thymidine at the various periods that I indicated; and this, of course, must have given a transient decrease in the concentration. Whether this accounts for the differences in our mortality and yours, I don't know.

However, the method of administering the gas may be of great importance. Releasing premixed gases from a cylinder under compression may produce erratic concentrations. Any system which utilizes such a method should be carefully analyzed during the period of administration.

M. Kuschner: Is it possible that the regional variation in response you have described might be secondary to regional variations in dose? It was my understanding that NO_2 effects might be maximal at the terminal bronchiole-respiratory bronchiole level because its limited solubility produces maximum "fall-out" at this level. Should this be so, the early, lesser degree of proliferation peripherally and its more prolonged persistence might simply be a reflection of more severe injury.

The second question I should like to raise relates to the estimation of secretory activity on the basis of numbers of secretory granules. I think you may have hinted at the answer. Is it not true that few granules might represent increased activity accompanied by increased discharge, or lesser activity accompanied by decreased formation.

Kleinerman: We designed the experiment in a way that I thought would obviate the local differences caused by differences in solubility; that is, in the acute experiments we exposed animals for at least 5½ hr. Now in a 5½-hr period, I would expect that not only is there a steady-state concentration throughout the entire lung, but also whatever local absorption might have occurred in the upper respiratory tract would have long since reached its maximum, so that after a short time there is an equal distribution throughout all

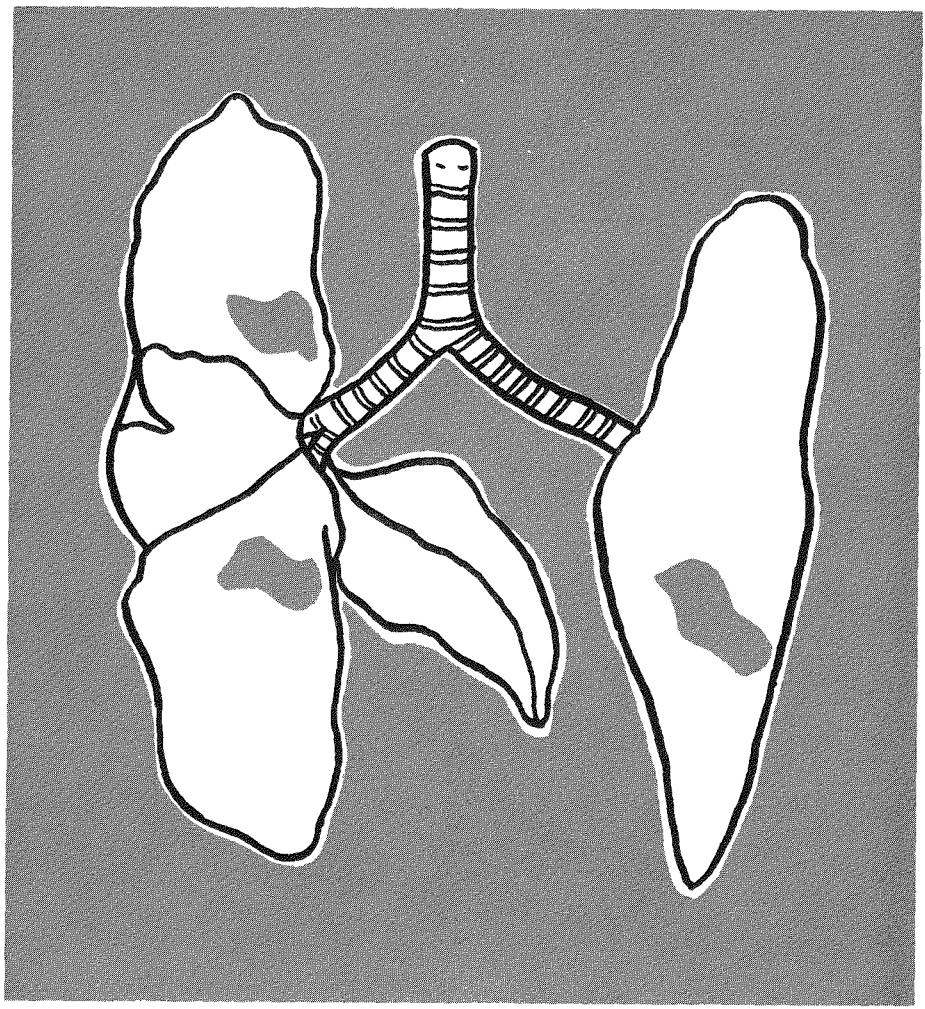
the portions of the respiratory tree. And the same is true with the chronic experiments. These are exposures of 22 hr per day, so I don't see how one could explain local differences on the basis of absorption or solubility. This same reactivity of the terminal-respiratory bronchiole region is so frequently seen associated with other types of injury that local solubility differences could not account for this general reaction.

It is possible that the numerical evaluation of the decrease in granules can be interpreted as increased production and release of granules or as a decrease in secretory activity. If the former alternative is considered there should be some evidence either on the lumenal aspect, extracellularly, or within the cell itself to indicate increased cell production of secretory granules – but this was not observed. This lack of evidence, plus the fact that the majority of cells counted did not contain increased amounts of secretory granules suggests strongly to me that the explanation favors decreased secretory activity.

SESSION III

RESPIRATORY CARCINOGENESIS

Chairman – T. Timothy Crocker
University of California
San Francisco Medical Center



LUNG CANCER: DOSE RESPONSE STUDIES WITH RADIONUCLIDES

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ABSTRACT

The induction of pulmonary carcinogenesis after deposition of radioactive particles depends on the amount and distribution of radiation dose in the lung. Nonuniform irradiation of the lung from radioactive particles is more carcinogenic than external irradiation. Similarly, for deposited particles, alpha irradiation is more carcinogenic than beta irradiation. "Point source" radioactive implants produce a high incidence of lung cancer. The doses required for a substantial tumor incidence are very high, when measured in proximity to the radioactive particle(s). – A model is derived which purports to estimate the carcinogenic risk associated with the inhalation of variously sized and activated particles from uranium-fueled reactors. Although the model establishes no threshold dose for carcinogenesis, it does provide an estimate of potential lung cancers that may result from deposition of single, large radioactive particles.

We should like to present first a review of studies in experimental animals which demonstrate a relationship between the distribution of radiation dose in the lung and the formation of lung cancers, and then to present a model for the estimation of pulmonary carcinogenesis from inhaled radioactive particles.

RADIOACTIVE PARTICLES AND PULMONARY CARCINOGENESIS

The development of lung cancer after exposure to radioactive particles has been discussed in several review articles.[1–4]

There are no data relating absorbed radiation dose and lung cancer incidence in man, other than the studies of uranium miners[5,6] and of survivors of atomic bombings in Japan.[7] Such epidemiological studies present evidence for an increased incidence of lung cancer with increasing radiation dose. For the most part, however, detailed evaluation of the carcinogenic hazards of inhaled radionuclides must of necessity be extrapolated from data obtained in experiments employing controlled populations of laboratory mammals.

Studies on the production of lung cancer by radionuclides may be conveniently subdivided into two categories: (1) those in which radioactive particles less than a few microns in diameter were injected intratracheally or inhaled and (2) those involving implantation of radioactive pellets, beads, or wires in the lung. Pertinent data – including type and amount of isotope, method of administration, latency period, species used, absorbed radiation dose, and incidence of lung cancer – are listed in Tables 1, 2, and 3.

Alpha Emitters (Category 1)

Of the micron-size particles tested, alpha emitters appear to be more carcinogenic than beta-gamma emitters on a comparable dose basis. Lung cancers in rats have been produced with ^{210}Po at average lung doses of less than 200 rads.[8] A moderate incidence of lung cancer (8 to 13%) was found after radiation doses in the lungs of a few thousand rads.[9-12] A 50 to 100% incidence of lung cancer was seen in rats living longer than 250 days after deposition of $^{239}PuO_2$.[13] No measurements of radiation dose were given in this report. A high incidence of lung cancer in dogs was observed after inhalation of $^{239}PuO_2$ particles.[14,15] Estimated lung doses ranged from 2500 to 13,600 rads. Dogs with the highest quantity of plutonium in their lungs died early, mostly from radiation-induced lung damage other than lung cancer. Fourteen of the 15 dogs that have died after surviving longer than 54 months have shown lung cancer.[16]

Beta-Gamma Emitters (Category 1)

The most useful body of data has come from studies in rats with ^{144}Ce,[17-20] most of which has been summarized by Cember.[3] Over 75% of the radiation dose was delivered to the lungs during the first 40 days after exposure.[17] Lung cancers were observed at mean lung doses as low as 680 rads (2% incidence) and as high as 68,000 rads (25% incidence). These results were similar to the lung cancer incidence observed in rabbits after pulmonary deposition of $^{144}CeF_3$ giving a maximum lung dose of 68,900 rads.[21]

Beta-Gamma Emitters (Category 2)

Disregarding dose considerations, radioactive devices (pellets, beads, or wires) implanted in the lung have been the most consistently effective means of producing radiogenic lung cancer in animals.[28-35,37-41] Death of animals surviving the trauma of implantation was often due to lung cancer rather than to more acute radiation effects, since a large fraction of the dose was absorbed in a small volume of the lung immediately surrounding the implant.[33,36] Implanted nonradioactive devices did not produce lung cancers. Data derived from these studies are shown in Table 3. Radiation doses of from 10^4 to 10^6 rads to lung tissue immediately surrounding the implants were required to induce lung cancer.

TABLE 1

*Data on Lung Cancer Incidence in Experimental Animals After Deposition of Radioactive Materials in the Lung: Alpha Emitters (Category 1)**

Reference	Species	Isotope	Quantity deposited (μCi, range)	Method of deposition	Estimated dose (rads)	Latency period (days, range)	Lung cancer incidence
8	Rat	^{210}Po-NaCl	0.02–0.15†	Inhalation	71	60–240	4
8	Rat	^{210}Po-NaCl	0.02–0.15†	Inhalation	202	60–240	10
8	Rat	^{210}Po-NaCl	0.02–0.15†	Inhalation	538	60–240	13
10	Rat	$^{210}Po(NO_3)_2$	5†	Tracheal	>2500	150–450	13
11	Mice	^{239}Pu citrate	0.06†	Tracheal	>1885	500	12
9, 12	Mice	$^{239}PuO_2$	0.06†	Tracheal	2300	400	8
13	Rat	$^{239}PuO_2$	0.2–1.0†	Tracheal		>250	"50–100"§
14	Dog	$^{239}PuO_2$	3.9–35.0‡	Inhalation	$9–23 \times 10^3$	150–1446	57
15	Dog	$^{239}PuO_2$	1.0–5.0‡	Inhalation	$2.5–13.6 \times 10^3$	1140–2370	60
16	Dog	$^{239}PuO_2$	1.0–5.0‡	Inhalation	$2.5–6.8 \times 10^3$	>1620	>90¶
22	Dog	$^{238}PuO_2$	17–261‡	Inhalation	$7.7–26.4 \times 10^3$	84	25

*Particles less than a few microns in diameter, administered by intratracheal injection or inhalation.
†Initial lung burden.
‡Alveolar lung burden.
§Estimated incidence in animals surviving longer than 250 days.
¶Incidence in dogs surviving more than 1620 days.

TABLE 2

*Data on Lung Cancer Incidence in Experimental Animals After Deposition of Radioactive Materials in the Lung Beta Gamma Emitters (Category 1)**

Reference	Species	Isotope	Quantity deposited† (μCi range)	Method of deposition	Estimated dose (rads)‡	Latency Period (days range)	Lung cancer incidence
23	Rat	^{198}Au colloid	11 150	Tracheal	5 4 8 0 × 10^3	70 360	10
23	Rat	$Cr^{32}Po_4$	40 100	Tracheal	14 15 × 10^3	180 540	14
23	Rat	$^{59}Fe_2O_3$	1 27	Tracheal	500 10 000	180 270	15
1	Mice	$Ba^{35}SO_4$	0 16	Tracheal	4000	100	2
24	Rat	$Ba^{35}SO_4$	375	Tracheal	12 20 × 10^3	312 319	13
9	Mice	$^{106}RuO_2$	1 9 3 0	Tracheal	4 9 × 10^3	350 422	5
18	Rat	$^{144}CeF^3$	0 5 50	Tracheal	6 5 × 10^2		2
18	Rat	$^{144}CeF^3$	0 5 50	Tracheal	1 4 × 10^3		3
18	Rat	$^{144}CeF^3$	0 5 50	Tracheal	4 6 × 10^3		5
18	Rat	$^{144}CeF^3$	0 5 50	Tracheal	1 0 × 10^4		10
18	Rat	$^{144}CeF^3$	0 5 50	Tracheal	2 0 × 10^4		13
18	Rat	$^{144}CeF^3$	0 5 50	Tracheal	4 0 × 10^4		23
18	Rat	$^{144}CeF^3$	0 5 50	Tracheal	4 8 × 10^4		25
17	Rat	$^{144}CeF^3$	5 50	Tracheal	2 4 × 10^3	48 178	4
17	Rat	$^{144}CeF^3$	5 50	Tracheal	5 1 × 10^3	48 178	4
17	Rat	$^{144}CeF^3$	5 50	Tracheal	1 1 × 10^4	48 178	25
17	Rat	$^{144}CeF^3$	5 50	Tracheal	2 1 × 10^4	48 178	27
21	Rabbit	$^{144}CeF^3$	25	Tracheal	5 1 6 9 × 10^4	238 327	25
19	Rat	$^{144}CeCl_3$	10 30	Tracheal	1 4 × 10^4	70 466	14§
19	Rat	$^{144}CeCl_3$	10 30	Tracheal	2 2 × 10^4	70 466	59§
19	Rat	$^{144}CeCl_3$	10 30	Tracheal	4 4 × 10^4	70 466	70§
25	Rat	^{59}Fe citrate oxide	1 06 27 5	Tracheal	490 9800	>180	
26	Rat	^{32}P	500	Tracheal		360 480	4
26	Rat	^{103}Ru	340 600	Tracheal			6
27	Rat	^{198}Au colloid	~150	Tracheal	9000	>90	12

*Particles less than a few microns in diameter administered by intratracheal injection or inhalation
†Initial lung burden
‡Integrated total lung dose
§Total number of tumors divided by total number of rats

TABLE 3

*Data on Lung Cancer Incidence in Experimental Animals After Deposition of Radioactive Materials in the Lung Beta Gamma Emitters (Category 2)**

Reference	Species	Isotope	Quantity deposited (μCi range)	Estimated dose (rads)†	Latency period (days range)	Lung cancer incidence
28	Rat	^{106}Ru pellets	0 008 13 6	$7\,4 \times 10^{2}$	315 433	0‖
28	Rat	^{106}Ru pellets	0 008 13 6	$3\,4 \times 10^{3}$	315 433	7‖
28	Rat	^{106}Ru pellets	0 008 13 6	$3\,6 \times 10^{4}$	315 433	22‖
28	Rat	^{106}Ru pellets	0 008 13 6	$4\,6 \times 10^{5}$	315 433	58‖
28	Rat	^{106}Ru pellets	0 008 13 6	$1\,6 \times 10^{6}$	315 433	66‖
29	Rat	^{106}Ru pellets	5 6	$3\,2 \times 10^{5}$	129 424	0¶
29	Rat	^{106}Ru pellets	5 6	$4\,5 \times 10^{5}$	129 424	11¶
29	Rat	^{106}Ru pellets	5 6	$5\,2 \times 10^{5}$	129 424	20¶
29	Rat	^{106}Ru pellets	5 6	$5\,5 \times 10^{5}$	129 424	50¶
29	Rat	^{106}Ru pellets	5 6	$6\,9 \times 10^{5}$	129 424	85¶
29	Rat	^{106}Ru pellets	5 6	$8\,0 \times 10^{5}$	129 424	82¶
29	Rat	^{106}Ru pellets	5 6	$8\,6 \times 10^{5}$	129 424	89¶
29	Rat	^{106}Ru pellets	5 6	$9\,2 \times 10^{5}$	129 424	100¶
30	Rat	^{106}Ru pellets	6 11 3		126 266	81¶
29	Rat	^{32}P pellets	0 2 20	$4\,0 \times 10^{3}$		0
29	Rat	^{32}P pellets	0 2 20	$4\,0 \times 10^{4}$		33
29	Rat	^{32}P Pellets	0 2 20	$4\,0 \times 10^{5}$		56
31 32	Rat	^{90}Sr capsules	27–62		90 450	90**
33	Rat	^{90}Sr beads		22 000‡	333 581	17
34	Mice	^{60}Co wires	170 250	$>2\,0 \times 10^{5}$‡	97 315	11††
35	Mouse	^{60}Co wires	70 636	261 000‡	180§	20
35	Rat	^{60}Co wires	70 636	353 000‡	204§	75
35	Hamster	^{60}Co wires	70–636	424 000‡	495§	8
35	Guinea Pig	^{60}Co wires	70 636	510 000‡	416§	25
35	Rabbit	^{60}Co wires	70 636	909 000‡	427§	42

*Implanted sources
†Dose to basal layer of bronchial epithelium 100 μm from implanted pellet
‡Method of dose calculation not adequately defined
§Median duration for animals with malignant lung tumors
‖For rats surviving longer than 143 days
¶For rats dying 129 to 424 days after implantation
**For rats surviving from 12 to 15 months after implantation
††For mice living longer than 97 days

RADIATION DOSE-CARCINOGENESIS RELATIONSHIPS

Considering the inadequacies in dose estimation which have characterized most of these studies, the absorbed radiation dose to the whole lung correlates rather well with the observed incidence of lung cancer following deposition of radioactive particles (Fig 1) In the case of both beta-gamma and alpha emitters, there is a rapid rise in the incidence of lung cancer with increasing log dose Unfortunately, data are not adequate to define "threshold doses" for induction of cancer – if, indeed, such thresholds exist

Uniform irradiation of the lung, in the form of external X- or gamma irradiation, is a relatively ineffective procedure for inducing lung cancers [42-50] If the large amount of energy deposited in a small volume of tissue surrounding a radioactive implant, which demonstrably can produce lung cancer, were distributed over the entire lung, little carcinogenic response would be expected

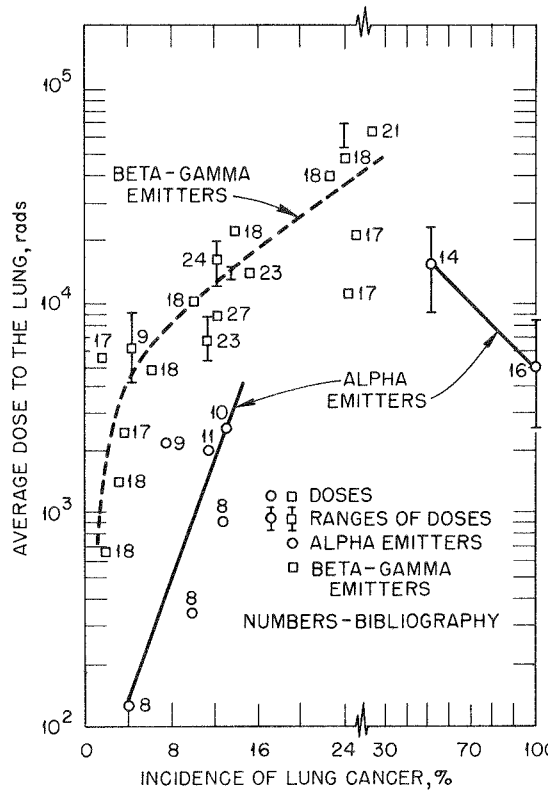

Fig 1 Relationships of total radiation dose to the lung after deposition of micron size or smaller beta, gamma, or alpha emitting particles, and the incidence of induced lung cancer (curves were drawn by eye).

Correspondingly, greater localization of energy around alpha-emitting particles results in more lung cancers, at lower total lung doses, than are found with beta-emitting particles The best means of obtaining a radiation dose high enough to induce lung cancer, without killing the animal from radiation pneumonitis, is to limit the irradiated volume to a functionally insignificant portion of the lung. This may be accomplished by utilizing large radioactive particles, by increasing the specific activity of a relatively small number of particles, or by implanting or injecting radioactive particles in a small volume of lung tissue.

A MODEL FOR THE ESTIMATION OF PULMONARY CARCINOGENESIS FROM INHALED NUCLEAR REACTOR DEBRIS

Extrapolation of the available experimental data on radiation-induced pulmonary cancer to the specific problem posed by the inhalation of radioactive particles released from nuclear rocket reactors involves a number of assumptions The particles are assumed to have originated from uranium-fueled reactors The reactor has undergone a maximal burn time assumed to be 10 min and, therefore, contains a substantial inventory of fission products [51] The particles are assumed to be spherical with varying diameters The radiation dose from a given particle will depend on the size of the particle and its fission product composition, which is a function of its decay time prior to deposition in the lung. Radiological aspects of this problem have been previously discussed in numerous reports [51-56,59-61] Doses used in the present study are from reference 52

The gamma component makes up a small portion of the total emitted energy from these particles. Essentially all the dose is from beta emissions in volumes near the particle. The average beta energies at increasing times after shutdown of the reactor are listed in Table 4. The beta dose at any given time is a function of

TABLE 4

*Average Beta Energy for 77 Nuclides Formed from "Spent" Uranium Reactor Fuel at Increasing Decay Times**

Time after shutdown (sec)	Average beta energy (Mev/disintegration)
10^3	1 160
10^4	0 850
10^5	0 509
10^6	0 291
10^7	0 316
10^8	0 462

*Data taken from reference 51

the emission rate (μCi) and the beta energy spectrum. The emitted beta energies from uranium carbide (UC_2) particles (Table 4) encompass the energy spectrum of radionuclides that have been utilized in experimental studies of the production of lung cancer in animals (Table 3.)

Tissue dose rates, calculated in rads per hour at a 100-μm distance from UC_2 particles, are presented in Table 5 for particles of various sizes, and at various times after reactor shutdown. The dose at 100 μm was used in this model since the only usable information on experimental lung cancer in animals after exposure to single, large radioactive sources relates to cancer incidence dose at 100 μm from bronchial implants of ^{106}Ru and ^{32}P.[28-29] The critical importance of particle size and decay time is evident from this tabulation.

TABLE 5

*Beta Dose Rates (rads/hr) at 100 μm, in Tissue, from Uranium Carbide Particles at Different Decay Times**

Particle diameter (μm)	Decay times (sec)					
	10^3	10^4	10^5	10^6	10^7	10^8
3.75	1.2×10^3	1.1×10^2	1.0×10^1	8.8×10^{-1}	5.9×10^{-2}	1.8×10^{-3}
15.0	6.3×10^4	5.9×10^3	4.9×10^2	4.3×10^1	2.6×10^0	8.4×10^{-2}
30.0	4.1×10^5	3.7×10^4	3.3×10^3	2.5×10^2	1.5×10^2	4.8×10^{-1}
60.0	2.4×10^6	2.2×10^5	1.8×10^4	1.2×10^3	7.2×10^1	2.5×10^0
120.0	1.2×10^7	1.0×10^6	7.7×10^4	4.9×10^3	1.9×10^2	1.0×10^1
240.0		3.4×10^6	2.2×10^5	1.3×10^4	8.5×10^2	3.1×10^1

*From reference 51, using the TDD dosimetry model.

Cumulative radiation doses from particles of various sizes, deposited in various lung compartments at various times after reactor shutdown, were calculated. Two models were employed for these calculations. The first (*static model*) was concerned with particles deposited and tenaciously retained in the alveoli. Doses were calculated for a 12-day period. It was assumed that the dose was accumulated in a geometrically unchanging volume of tissue. The second (*mobile model*) was concerned with particles deposited in the tracheo-broncheo-bronchiolar compartments, from which the particles may be assumed to be cleared within the first day after deposition. The radiation dose will be delivered to a strip of epithelium corresponding to the upward movement of the clearing particle.

Clearance phases and times as determined by Morrow *et al.*[57] in man were utilized in these calculations. The parameters employed are presented in Table 6, which relates cumulative times and rates for particles deposited in the various anatomical compartments. The farther the penetration of the radioactive particle in the pulmonary branching system, the slower the clearance and the greater the radiation dose to the epithelium.

TABLE 6

Length of Human Airways as Related to the Generation of Airway Branching and Particle Clearance Phases and Clearance Times*

Airway-branching generation number	Structural compartment	Cumulative length (mm)	Clearance phase†	Clearance time‡	Linear clearance rate§ (mm/min)
0	Trachea	120.0	Tracheal	6 min	20
1–3	Bronchi	4.2	Upper bronchial	60 min	1.2
4–16	Bronchioles	72.2	Phase A	240 min	0.3
17–19	Terminal bronchioles and alveolar ducts	5.7	Phase B	600 min	0.01
20+	Alveoli	0.050	Phase C	100 days	

*From Weibel's[58] regular dichotomy lung model.

†The clearance phases are estimates for man from data by Morrow *et al.*[57]

‡The clearance times are estimated absolute values, based on the biological half-lives of radioactive particles in man,[57] and represent the clearance times of particles from each structural compartment. The particle is assumed to be cleared upward over the shortest possible distance up each generation branch, comprising the entire structural compartment in question.

§The linear clearance rate is the distance the particle moves upward in 1 min and is calculated by dividing the time for clearance in that structural compartment into the cumulative length of each structural compartment.

Particles are assumed to have been inhaled and deposited at 10^3 or 10^4 sec after reactor shutdown. For the mobile model, dose was calculated for every millimeter of distance the particle travels up the bronchioles, bronchi, and trachea and for every tenth micrometer of distance the particle travels up the alveolar ducts and terminal bronchioles. Dose rates in tissue at 100 μm from UC_2 particles (Table 5) were fitted by least squares regression to the mathematical expression, dose rate = A $(\text{time})^{-B}$, with time expressed in seconds after shutdown of the reactor. The computer program calculated the dose received by the epithelium from the equation shown below:

$$\int \text{dose rate} = A \int_{T_1}^{T_2} T^{-B}\, dT\,,$$

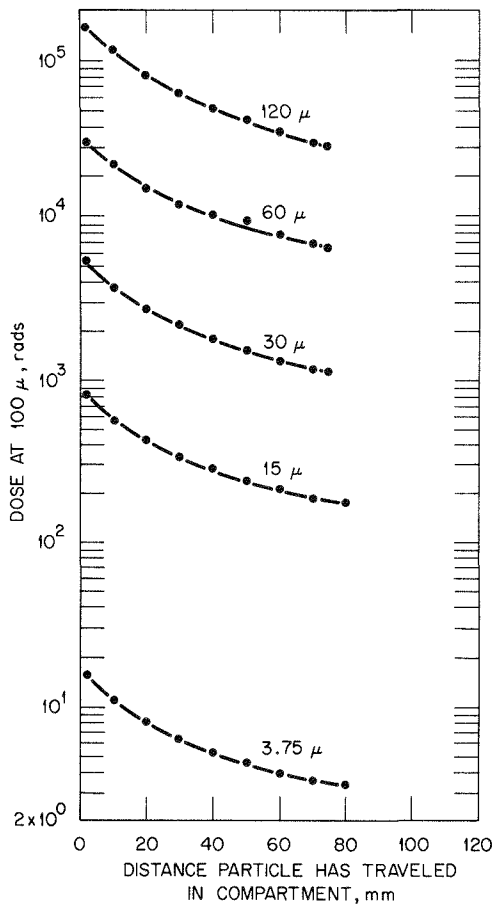

Fig. 2 – Radiation dose delivered to the bronchi by uranium carbide particles (10^3 sec decay time) of various diameters being cleared from the lung (mobile model).

or

$$\text{dose} = \frac{A}{1-B}\left[T_2^{\,1-B} - T_1^{\,1-B}\right]$$

Examples of the results of these calculations are shown in Figs. 2 and 3. A summary of "average" doses received by the epithelium in each respiratory compartment, for each particle size considered, and for the two decay periods considered, is given in Table 7.

As would be expected from the clearance rates, the lower respiratory passages received considerably higher doses than the upper respiratory passages. Clearing particles $\leqslant 3.75\ \mu$ in diameter resulted in most cases in doses of <100 rads. No

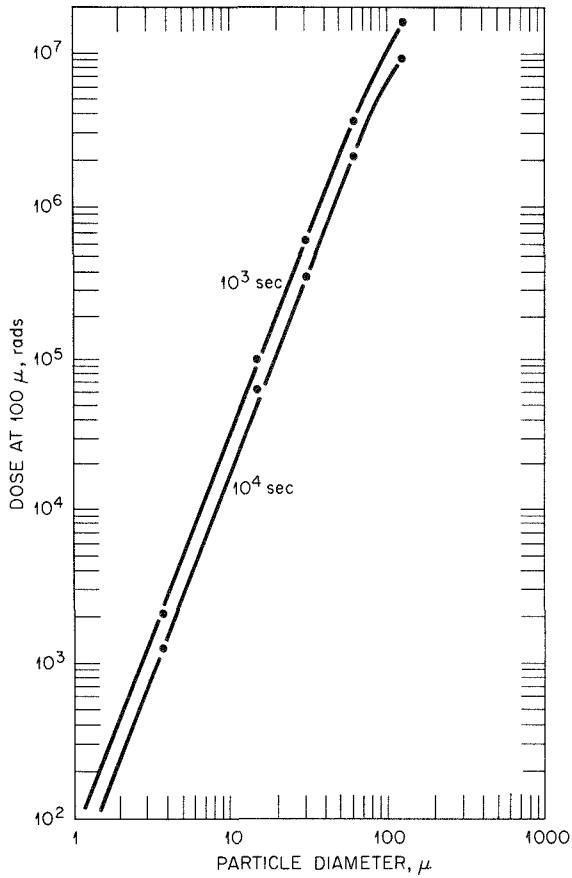

Fig. 3 – Radiation dose delivered to the alveoli after deposition of uranium carbide particles (static model).

TABLE 7

An Educated Guess as to Incidence of Lung Cancer After Radiation Doses to the Pulmonary Epithelium from Clearing and Static Uranium Carbide Particles

Compartment	Particle size (μm)	Decay time at which particle is deposited (sec)	Average dose to epithelium at 100-μm depth (rads)	Estimated incidence of lung cancer (%)
Trachea	3.75	10^3	0.8	
Trachea	15.0	10^3	45	
Trachea	30.0	10^3	290	
Trachea	60.0	10^3	1800	
Trachea	120.0	10^3	8900	13
Trachea	3.75	10^4	0.08	
Trachea	15.0	10^4	4.6	
Trachea	30.0	10^4	29	
Trachea	60.0	10^4	170	
Trachea	120.0	10^4	780	
Bronchi	3.75	10^3	6.7	
Bronchi	15.0	10^3	360	
Bronchi	30.0	10^3	2300	
Bronchi	60.0	10^3	14,000	17
Bronchi	120.00	10^3	66,000	34
Bronchi	3.75	10^4	1.2	
Bronchi	15.0	10^4	64	
Bronchi	30.0	10^4	410	
Bronchi	60.0	10^4	2300	
Bronchi	120.0	10^4	11,000	15
Bronchioles	3.75	10^3	12	
Bronchioles	15.0	10^3	630	
Bronchioles	30.0	10^3	4000	4
Bronchioles	60.0	10^3	24,000	23
Bronchioles	120.0	10^3	110,000	40
Bronchioles	3.75	10^4	3.6	
Bronchioles	15.0	10^4	190	
Bronchioles	30.0	10^4	1200	
Bronchioles	60.0	10^4	6800	9
Bronchioles	120.0	10^4	31,000	26
Terminal bronchioles and alveolar ducts	3.75	10^3	20	
Terminal bronchioles and alveolar ducts	15.0	10^3	1000	
Terminal bronchioles and alveolar ducts	30.0	10^3	6500	9
Terminal bronchioles and alveolar ducts	60.0	10^3	38,000	28
Terminal bronchioles and alveolar ducts	120.0	10^3	160,000	44
Terminal bronchioles and alveolar ducts	3.75	10^4	7.8	

TABLE 7 (continued)

Compartment	Particle size (μm)	Decay time at which particle is deposited (sec)	Average dose to epithelium at 100-μm depth (rads)	Estimated incidence of lung cancer (%)
Terminal bronchioles and alveolar ducts	15 0	10^4	400	
Terminal bronchioles and alveolar ducts	30 0	10^4	2500	
Terminal bronchioles and alveolar ducts	60.0	10^4	14,000	17
Terminal bronchioles and alveolar ducts	120 0	10^4	63,000	33
Alveoli	3 75	10^3	2000	
Alveoli	15 0	10^3	100,000	38
Alveoli	30 0	10^3	640,000	>50
Alveoli	60.0	10^3	3,600,000	>50
Alveoli	120.0	10^3	16,000,000	>50
Alveoli	3.75	10^4	1300	
Alveoli	15 0	10^4	64,000	33
Alveoli	30 0	10^4	400,000	>50
Alveoli	60 0	10^4	2,100,000	>50
Alveoli	120 0	10^4	9,300,000	>50

*Lung cancer estimates obtained by interpolation from data presented in Table 3 (refs 28, 29) See text for assumptions involved in such estimates

lung cancers would be expected from such doses One past investigation is of particular interest in this regard. Radioactive particles of about 1000-μm diameter were obtained from the Trinity Test Site and implanted in the lungs of 100 rats The beta dose received in "the first few millimeters" around each particle was calculated as 340 rep delivered in a 12-month period. No evidence of malignant change was observed. The authors concluded that "the amount of radioactivity used was too little and the time of exposure was too short to induce neoplastic changes in surrounding lung tissue."[41]

Included in the last column of Table 7 are "estimates" of lung cancer incidence to be expected from the listed doses These estimates are based on the experimental dose-effect relationships presented in Table 3,[28,29] and must be considered highly speculative in view of the many assumptions involved in such extrapolation Some of the more critical assumptions include (1) that man is similar to the rat in carcinogenic potential relative to radiation dose, (2) that the carcinogenic potential of the epithelium is similar throughout all respiratory compartments, (3) that the carcinogenic potential of a given radiation dose is independent of the rate at which this dose is delivered, and (4) that the carcinogenic potential is uninfluenced by age, sex, prior exposure to other

carcinogens, and a wide variety of other physiological and environmental factors not taken into consideration.

In view of the many uncertainties involved we feel that it is completely unjustified to infer numbers for tumor incidence at doses lower than 3000 rads. For the same reason, any tabulated compilation of numbers purporting to combine the probabilities of inhalation of particles with the probabilities of internal deposition of particles, and with the probabilities that cancer will result from such deposited particles, can only be considered a mathematical exercise that obscures the most important fact – that the data necessary for any overall evaluation of the hazard simply do not exist. The kinetics of deposition and retention of inhaled particles is a critical factor in the overall hazard evaluation problem – particularly as it applies to particles larger than about 10-μm diameter, which is usually considered the upper limit of respirability. It is only these very large particles that are capable of delivering a radiation dose of significant carcinogenic potential, and although the probability of their deposition may be very small, it is necessary to know how small before the probability can be disregarded. Such data on the respirability of particles in the 10- to 50-μ size range should be obtained. In view of the very low deposition figures to be anticipated, such data would have little relevance unless obtained in humans.

CONCLUSIONS

Radiation can produce lung tumors. Uniform irradiation appears to be relatively ineffective in this regard, since pathology other than neoplasia predominates at radiation levels which produce a substantial effect of any kind. However, it cannot be deduced from presently available data that a very low incidence of lung tumors might not result from a radiation dose too low to result in other serious effects.

Nonuniform irradiation of the lung from deposited radioactive particulates is clearly more carcinogenic than uniform exposure (on the basis total lung dose), and alpha irradiation is more carcinogenic than beta irradiation. The doses required for a substantial tumor incidence are very high, however, if measured in proximity to the particle; and, again, there are no data to establish the low-incidence end of a dose-effect curve. And there is no general theory, or data on which to base a theory, which would permit extrapolation of the high incidence portion of the curve into the low incidence region.

Such data could be obtained and is very urgently needed. It would relate tumor incidence to radiation dose over a range of doses, with a sufficient number of animals of more than one species. It would compare the effects of different radiation qualities – gamma vs. beta vs. alpha. It would compare the effects of different dose distributions – uniform exposure vs. many particulate sources vs. a single "hot" particle. It would compare the effects of protracted vs. short-term irradiation. It would be a costly experiment of many years duration.

It would provide not only a direct experimental simulation of the types of exposure which might be expected to occur in a disaster situation, but, hopefully, it would also lead to a total picture of radiation carcinogenesis in the lung which would permit the establishment of a general theory on the basis of which confident predictions might be made concerning situations not specifically tested.

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REFERENCES

1. Report of the Subcommittee on Inhalation Hazards, Effects of Inhaled Radioactive Particles, Publication No. 848. National Academy of Sciences, National Research Council, Washington, D.C., 1961.
2. J. N. Stannard, An evaluation of inhalation hazards in the nuclear energy industry. In, *Second International Conference on the Peaceful uses of Atomic Energy,* Vol. 23, p. 306. United Nations, Geneva, Switzerland, 1958.
3. H. Cember, Radiogenic lung cancer. In, *Progress in Experimental Tumor Research,* Vol. 4, p. 251, F. Homburger (ed.). Hafner, New York, 1964.
4. W. J. Bair, Inhalation of radionuclides and carcinogenesis, these *Proceedings,* p. 77.
5. Federal Radiation Council, Radiation Exposure of Uranium Miners, Report of an Advisory Committee from the Division of Medical Sciences, National Academy of Sciences – National Research Council – National Academy of Engineering, Washington, D.C., August 1968.
6. Federal Radiation Council, Guidance for the Control of Radiation Hazards in Uranium Mining, Staff Report No. 8, Revised. Superintendent of Documents, U.S. Government Printing Office, Washington, D.C., September 1967.
7. C. K. Wanebo, K. G. Johnson, K. Sato, and T. W. Thorslund, Lung cancer following atomic radiation. *Am. Rev. Respirat. Diseases,* 98 (1968) 778.
8. C. L. Yuile, H. L. Berke, and T. Hull, Lung cancer following polonium-210 inhalation in rats. *Radiation Res.,* 31 (1967) 760.
9. S. A. Temple, S. A. Marks, and W. J. Bair, Tumors in mice after pulmonary deposition of radioactive particles. *Intern. J. Radiation Biol.,* 2 (1960) 143.
10. J. K. Scott and R. G. Thomas, Induction of pulmonary tumors following intratracheal injection of polonium-210. *University of Rochester Atomic Energy Project Quarterly Review,* July-September (1957) 30.
11. R. Wager, N. L. Dockum, L. A. Temple, and D. H. Willard, Toxicity of radioactive particles. IA. Intratracheal injection of radioactive suspensions. In, Biology Research Annual Report for 1955, p. 61. Available from AEC Division of Technical Information Extension, Oak Ridge, Tennessee.
12. L. A. Temple, D. H. Willard, S. Marks, and W. J. Bair, Induction of lung tumors by radioactive particles. *Nature,* 183 (1959) 408.
13. H. Lisco, Autoradiographic and histopathologic studies in radiation carcinogenesis of the lung. *Lab. Invest.,* 8 (1959) 162.
14. W. J. Clarke, J. F. Park, J. L. Palotay, and W. J. Bair, Bronchiolo-alveolar tumors of the canine lung following inhalation of plutonium particles. *Am. Rev. Respirat. Diseases,* 90 (1964) 963.

15. J. F. Park, W. J. Bair, W. J. Clarke, and E. B. Howard, Chronic effects of inhaled plutonium dioxide in dogs. In, Pacific Northwest Laboratory Annual Report for 1966, Vol. 1, Biological Sciences, BNWL-480, p. 57. Battelle-Northwest, Richland, Washington.
16. J. F. Park, E. B. Howard, and W. J. Bair, Chronic effects of inhaled $^{239}PUO_2$ in beagles. In, Pacific Northwest Laboratory Annual Report for 1968, BNWL-1050, Vol. I, Part 1. Battelle-Northwest, Richland, Washington, in press.
17. H. Cember, J. A. Watson, and A. A. Spritzer, Bronchogenic carcinoma from radioactive cerium fluoride. *A.M.A. Arch. Ind. Health,* 19 (1959) 14.
18. H. Cember, Emperical establishment of cancer-associated dose to the lung from ^{144}Ce. *Health Phys.*, 10 (1964) 1177.
19. H. Cember and K. Stemmen, Lung cancer from radioactive cerium chloride. *Health Phys.* 10 (1964) 43.
20. H. Cember, Further studies on lung cancer from Ce^{144} F_3. *Health Phys.* 9 (1963) 539.
21. N. N. Kurshakova and A. E. Ivanov, A model of experimental lung cancer caused by intratracheal introduction of radioactive cerium. *Bull. Exptl. Biol. Med.*, 54 (1962) 787.
22. J. F. Park, E. B. Howard, and W. J. Bair, Acute toxicity of inhaled $^{238}PUO_2$ in beagles. In, Pacific Northwest Laboratory Annual Report for 1968, BNWL-1050, Vol. I, Part 1. Battelle-Northwest, Richland, Washington, in press.
23. T. A. Kochetkova, G. A. Avrunina, and N. D. Sagaidak-Chernyak, Experimental lung cancer induced with radioactive compounds P^{32}, Au^{198}, and Fe^{59}. *Acta Unio. Intern. Contra. Cancrum.*, 19 (1963) 684.
24. H. Cember and J. A. Watson, Bronchogenic carcinoma from radioactive barium sulfate. *A.M.A. Arch. Ind. Health,* 17 (1958) 230.
25. N. D. Sagaidak and M. Potoksikol, Morphological changes in rats after intratracheal introduction of various iron-59 compounds. *Radioaktivn. Veschestv. Inst. Gigieny Trudai Profze-bolevanii Akad. Med. Nauk USSR,* 3 (1962) 131. (NSA 18, 6720, 1964)
26. L. A. Grizute, On the experimental cancer of the lung. In, *Proceedings of the 4th Session of the Oncological Institute, Lit. USSR Vilnius,* p. 53.
27. T. A. Kochetkova and G. Avrunina, Veranderungen in den Lungen und in anderen Organen bec intratrachealev Einfuhrung einiger Radioisotope. *Arch. Gewerbepathol. Gewerbehyg.*, 16 (1957) 24.
28. S. Laskin, M. Kuschner, N. Nelson, B. Altshuler, J. H. Harley and M. Daniels, Carcinoma of the lung in rats exposed to the beta-radiation of intrabronchial ruthenium-106 pellets. I. Dose-response relationships. *J. Natl. Cancer Inst.*, 31 (1963) 219.
29. S. Laskin, M. Kuschner, B. Altshuler, and N. Nelson, Tissue reactions and dose relationships in rats following intrapulmonary beta radiation. *Health Phys.* 10 (1964) 1229.
30. M. B. Divertie, J. L. Titus, and R. G. Shorter, A simple method for the production of experimental squamous cell carcinoma of the lung in rats. *Am. Rev. Respirat. Diseases,* 96 (1967) 820.
31. H. W. Altmann, W. Hunstein, and E. Stutz, Strahleuinduzierte (Sr^{90}) Lungencarcinome bei Ratten. *Naturwissenschaften,* 46 (1959) 85.
32. H. W. Altmann, W. Hunstein, and E. Stutz, Uber Lungenveranderungan und Lungentumoreu bei Ratten nach Bestrahlung mit radioaktiven Stroutium (Sr^{90}). *Beitr. Pathol. Anat. Allgem. Pathol.*, 124 (1961) 141.
33. H. Cember and J. A. Watson, Carcinogenic effects of strontium-90 beads implanted in the lungs of rats. *Am. Ind. Hyg. Assoc. J.*, 19 (1958) 36.
34. O. Gates and S. Warren, The production of bronchial carcinoma in mice. *Am. J. Pathol.*, 36 (1960) 653.

35 S Warren, and O Gates Cancers induced in different species by continuous gamma-radiation *Arch Environ Health* 17 (1968) 697
36 B Altshuler, Dosimetry from a Ru^{106}-coated platinum pellet *Radiation Res* 9 (1958) 626
37 O Gates and S Warren, Histogenesis of lung carcinoma in mice, induced by gamma radiation *Arch Pathol* 71 (1961) 693
38 H W Altmann, R Lick, and E Stutz, Uber die Histogenese strahleninduzierter (Sr^{90}) Plattenepithelcarcinome in der Rattenlungen II Die Rolle des Alveolarepithels *Beitr Pathol Anat Allgem. Pathol* 125 (1961) 427
39 M Kuschner, S Laskin, E Christofano, and N Nelson, Experimental carcinoma of the lung In, *Proceedings of the Third National Cancer Conference* p 485 J B Lippincott Co , Philadelphia, Pa , 1956
40 N Nelson, Radiation cancer of the lung tissue sensitivity and factors influencing dosage from airborne radioactive substances In, *Inhaled Particles and Vapours* p 221, C N Davies (ed) Pergamon Press, N Y , 1961
41 G V Taplin, O M Meredith, and H Kade, Evaluation of the Acute Inhalation Hazard from Radioactive Fallout Material by Analysis of Results from Field Operations and Controlled Inhalation Studies in the Laboratory, Preliminary Report on Operation Teapot, Nevada Test Site, Project 37 3 University of California, Los Angeles, California, 1955
42 S Warren and O Gates, Radiation pneumonitis experimental and pathological observations *Arch Pathol* 30 (1940) 440
43 J C Smith, Review of Radiation Pneumonitis, WRV-110 Western Reserve University, Cleveland, Ohio
44 A C Andersen and P Guttman, Lung neoplasms in whole-body x-irradiation beagles In, *Lung Tumors in Animals* p 359, L Severi (ed) University of Perugia, Italy
45 J Furta and O B Furth, Neoplastic disease produced in mice by general irradiation with x-rays I Incidence and types of neoplasms *Am. J Cancer* 28 (1963) 54
46 H I Kohn, M L Epling, P H Guttman, and D W Bailey, Effect of paternal (spermatogonial) x-ray exposure in the mouse life-span, x-ray tolerance and tumour incidence of the progeny *Radiation Res* , 25 (1965) 423
47 B G Lamson, M S Billings, and L R Bennett, Incidence of disease and Cause of Death Following Single and Divided Doses of Total Body X-Irradiation in Rats, UCLA-12-617 University of California, Los Angeles, California
48 J H Rust, R J Robertson, E F Staffeldt, G A Sacher D Grahn, and R J M Fry, Effects of lifetime periodic gamma-ray exposure on the survival and pathology of guinea pigs In, ANL-7136, p 24 Argonne National Laboratory, Argonne, Illinois, 1965
49 J Hallcroft, Carcinogenesis of chronic gamma irradiation In, *Radiation Biology and Cancer* p 409 University of Texas Press, Austin, Texas, 1959
50 E Lorenz *et al* Effects of long-continued total body gamma irradiation on mice, guinea pigs, and rabbits, III Effects on life-span, weight, blood picture and carcinogenesis and the role of intensity of radiation In, *Biological Effects of External X and Gamma Radiation,* National Nuclear Energy Series IV-22B, p 24, R E Zirkle (ed) McGraw-Hill, New York, N Y , 1954
51 J C Ulberg and D B Kochendorfer, Models for Estimating Beta Dose to Tissue from Particle Debris in Aerospace Applications, USNRDL-TR-1107 U S Naval Radiological Defense Laboratory, San Francisco, California, 1966
52 S A Mikhail and K A Collins, Analysis of the Particulate Effluent Data from Phoebus IB EP-IV Nuclear Reactor Test USNRDL-TR-68-23 U S Naval Radiological Defense Laboratory, San Francisco, California, 1968

53. J. S. Krebs, The Response of Mammalian Skin to Irradiation with Particles of Reactor Debris, USNRDL-TR-67-118. U.S. Naval Radiological Defense Laboratory, San Francisco, California, 1967.
54. D. B. Kochendorfer and J. C. Ulberg, Human Exposure to Particulate Debris from Aerospace Nuclear Applications, USNRDL-TR-67-59. U.S. Naval Radiological Defense Laboratory, San Francisco, California, 1967.
55. B. R. Fish, T. G. Clark, R. H. Boyett, W. H. Wilkie, Jr., and J. L. Thompson, Environmental Studies: Radiological Significance of Nuclear Rocket Debris, Progress Report for the Period July 1, 1965–June 30, 1966, ORNL-TM-1686. Oak Ridge National Laboratory, Oak Ridge, Tennessee, 1966.
56. J. F. Pestaner, D. A. Kubose, and W. R. Balkwell, Beta Dose Rates from Neutron-Irradiated Uranium-235 Carbide Particles, USNRDL-TR-1082. U.S. Naval Radiological Defense Laboratory, San Francisco, California, 1966.
57. P. E. Morrow, R. F. Gibb, and K. M. Gazioglu, A study of particulate clearance from the human lungs. *Am. Rev. Respirat. Diseases,* 96 (1967) 1209.
58. E. R. Weibel, *Morphometry of the Human Lung,* p. 139. Academic Press, New York, 1963.
59. A. Kirschbaum, Relation of radiation carcinogenesis to carcinogenesis induced by other means. In, *Radiation Biology and Cancer,* p. 303. University of Texas Press, Austin, 1959.
60. J. Vaughan, Non-uniformity of radiation dose in space with special reference to radiological protection. *Intern. J. Radiation Biol.,* 9 (1965) 513.
61. J. S. Bevan and A.K.M.M. Haque, Some speculations on the carcinogenic effect of inhaled alpha-active material. *Phys. Med. Biol.,* 13 (1968) 105.

DISCUSSION

G. Poda: Has any attempt been made to determine whether or not these lesions can be found macroscopically? In other words, have the animals been X-rayed before killing, or anything like that?

C. L. Sanders: No, we have not examined radiographs in our rats. Dr. Jim Park has been routinely examining plutonium-exposed dogs; perhaps he could comment.

J. F. Park: We have not radiographed any of the lesions in the rats, but we have examined the radiographic lesions in dogs, where the tumors have been induced by inhaled plutonium. In these cases, the lesions can be detected as early as a year and a half before sacrifice of the animal.

R. O. McClellan: My comment relates to the use of bronchopulmonary lavage as therapy for removal of the inhaled particulate material. I think this represents a highly significant and potentially very useful therapeutic method for accidental inhalation exposures; therefore, I would like to expand briefly on your comments.

In a study conducted in our laboratory [R. C. Pfleger *et al., Dis. Chest,* in press], beagles were lavaged after having been exposed to aerosols of ^{95}zirconium-^{95}niobium oxide. They were lavaged on the day of exposure, day 8 postexposure, day 16 postexposure, or on all three of those days. We lavaged the

entire left lung with isotonic physiological saline. The most significant results, perhaps as expected, were obtained in those animals lavaged at all three time intervals, in which case half of the initial activity deposited in the left lung was removed, as compared to their right lung, resulting in a significant reduction in the radiation dose to the lung and, hopefully, a reduction in potential risk associated with inhaling the radioactive material.

Your work on estimation of dose, using the topographic technique, I find very interesting and I think will be proved to be quite fruitful. However, I question the method of fixation and preparation of your material, since I am certain that the results will be highly dependent upon the morphology of the fixed tissue. In your material it appeared that the lung was at least partially collapsed, which I think would lead to a high estimation of radiation dose. I would suggest you consider using a perfusion technique to fix the lungs in an inflated state.

Sanders: No. We ran this information through the computer and looked at the density of the lung expressed as the ratio of the surface area versus the surface area of the air space and found a normal lung density. Small tissue samples were removed from animals and fixed in glutaraldehyde prior to embedding in Epon 812. Calculation of rat alveolar diameter showed no apparent alveolar collapse.

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EFFECTS OF CHRONIC EXPOSURE TO ARTIFICIAL SMOG AND CHROMIUM OXIDE DUST ON THE INCIDENCE OF LUNG TUMORS IN MICE

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ABSTRACT

Specific-pathogen-free C57BL/6 mice, pretreated with 100 R of whole-body X-radiation and/or infected with PR8 influenza virus, were placed in inhalation chambers and exposed to either ozonized gasoline or an insoluble chromium oxide dust. The main findings were: male mice had a significantly higher incidence of lung tumors than females; irradiation raised the incidence of lung tumors in females but not in males; chronic exposure to ozonized gasoline increased the lung tumor incidence in both sexes; infection with PR8 influenza virus appeared to retard lung tumor growth; exposure to chromium oxide dust had no discernible effect on lung tumor incidence. The only lung tumors observed during the course of these experiments were adenomas and adenocarcinomas. Under the experimental conditions used, the combination of treatments had no additive or potentiating effects.

This study was initiated to determine the role of air pollutants in the induction of pulmonary tumors and to develop an experimental model for testing the carcinogenic and cocarcinogenic activity (for the lung) of various chemical and biological agents. Chronic inhalation was chosen as the *principal* mode of exposure in order to induce pathophysiological processes similar to those that develop in the human respiratory system in response to inhalation of noxious and potentially carcinogenic chemicals. A major question underlying the experimental design described below was: Does an antecedent injury of the lung modify the tissues of the respiratory tract and thereby increase their susceptibility to the tumor-inducing activity of inhaled particulate and gaseous chemicals. In the light of previous investigations in which squamous cell

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carcinomas were observed in mice as a consequence of viral pneumonitis and smog exposure,[1,2] particular attention was given to the possible interaction of infectious and chemical agents in the development of respiratory tumors.

Germfree-derived, specific-pathogen-free mice of the C57BL/6 inbred line were selected as experimental animals for this study. This allowed us to work with a large genetically homogeneous animal population free of most infectious agents. The C57BL/6 strain was selected because of its low spontaneous lung tumor incidence.

The following agents were chosen for the study: artificial smog (ozonized gasoline), because epidemiological and experimental evidence suggests that it might be an atmospheric pollutant with carcinogenic activity (for discussion see ref. 3); chromate dust, because it is an occupational hazard implicated as a carcinogen in man,[4] although experimental attempts to show its carcinogenicity on the respiratory tract have been largely unsuccessful;[5,6] X-radiation, because of its amply documented carcinogenic effects on respiratory tissues;[7,8] and PR8 influenza virus, because of previous studies indicating that its interaction with chemical carcinogens might lead to a morphologic type of lung tumor commonly observed in humans.[1,2]

EXPERIMENTAL DESIGN

The main experiment diagrammed in Table 1 involves three types of inhalants: filtered air, Cr_2O_3 dust, and artificial smog. For each of these, two inhalation chambers, each containing 545 mice in 45 wire cages, were used. In all six chambers half of the animals were pretreated at 4 weeks of age with a single whole-body exposure to 100 R of X-radiation, 4 weeks prior to inhalation exposure. In three chambers all animals were also infected with PR8 influenza

TABLE 1

Experimental Design

A. Inhalation chamber experiment

Group 1. Filtered air 2. Cr_2O_3 3. Smog	100 R / Unirradiated	♂ / ♀
4. Filtered air + PR8 5. Cr_2O_3 + PR8 6. Smog + PR8	100 R / Unirradiated	♂ / ♀

B. Nonchamber experiment

Group 7. No treatment 8. 100 R 9. PR8 10. 100 R + PR8	♂ / ♀

virus 2 weeks prior to the beginning of the inhalation experiment. Only mice that showed serological evidence of infection during the 2nd week were used in the subsequent study. Two weeks after the infection, when the acute pneumonitis had subsided, exposure to one of the three designated atmospheres began with daily exposures of 5½ hr a day, 5 days a week. At 6, 12, and 18 months after the start of the experiment, 15 mice were removed from every chamber and were used for bacteriological, virological, and parasitological testing, and for histopathological investigation.

Four other groups of mice, each consisting of 545 mice (one-half males and one-half females), were also set up. These were *not* put into the inhalation chambers, but were maintained in the "regular" mouse room environment within the barrier facility and lived in normal mouse cages with bedding. They received either no treatment, whole-body X-irradiation, PR8 influenza virus, or X-irradiation and virus.

Ten experimental groups were used in all. Groups 1–6 comprised the inhalation chamber experiment and Groups 7–10 the nonchamber experiment (see Table 1 for the treatments associated with these groups).

MATERIALS AND METHODS

Animals

The experimental design and nature of the inhalation program required animals that were free of mouse pathogens, internal and external parasites, and most common murine viruses. The inability to obtain such animals from commercial sources on a regular basis prompted us to establish a specific-pathogen-free (SPF) breeding colony.[9] The initial stock was supplied by the gnotobiotic animal facility of the Biology Division of the Oak Ridge National Laboratory. The mice were offspring of a C57BL/6 stock that was brother-sister mated for four generations, rederived by cesarean section into a germfree facility, and foster-nursed by ICR germfree females. This rederived stock was transferred into SPF barrier facilities. The breeder and research animals were routinely monitored for latent murine viruses, as well as for *Pseudomonas* sp., *Salmonella* sp., external and internal parasites, and mycoplasma. Throughout the course of the experiment, research animals were also routinely monitored for these agents. The history of the barrier facility shows occasional detection of Reo-3 virus, GD VII virus, and nonpigmented pseudemonades.

All animals were fed a balanced diet of Purine Pure-pack chow. Water was chlorinated at 15 parts per million.

Virus

The strain of influenza virus used in these studies was PR8-34-60 (obtained from the School of Public Health, University of Michigan, Ann Arbor). It was isolated in 1934 and has since been passed in Webster strain mice. We adapted it

to C57BL/6 mice for these experiments. This strain, which has an LD_{50} of $1 \times 10^{-6.2}$, was tested and found to be free of resistance-inducing factor (RIF-free).

We aerosolized the virus with a nebulizer that generated the majority of particles in the 1-μ range. Up to 345 animals were simultaneously exposed to the aerosol in one of our inhalation exposure chambers. Five milliliters of PR8 virus were generated over approximately a 30-sec period. Fans within the chamber circulated the virus throughout the chamber. After 30 min the chamber containing the exposed animals was flushed with filtered air for 2 hr; then the animals were removed and placed in filter-top cages.

Two weeks after infection all animals were orbitally bled and the HI titer of the serum determined. In all cases better than 90% of all animals were HI positive, with only 5% of the animals dying from acute pneumonitis during the 2-week post-infection holding period. Only animals that were HI positive were used for the inhalation experiment. Animals killed for base-line pathology, prior to exposure to the chemical carcinogen, proved to have lung consolidation, confirming the HI serology.

Irradiation

Animals were exposed to 100 R of X-radiation from a General Electric Maxitron 300 machine. The irradiation conditions were as follows: 300 kvp, 20 ma; 100 R/min; inherent filtration, 4.75 mm of Be; added filtration, 3 mm of Al, half-value layer (hvl), 0.5 mm Cu. The animals were irradiated in a circular perforated lucite container that was attached to a revolving turntable.

Inhalation Exposure

The inhalation exposure facility consisted of eight $3' \times 3' \times 5'$ stainless steel and plexiglass chambers with pyramidal tops and bottoms; ten changes of filtered air at 71° to 75°F, and 45-55% relative humidity, were supplied to the chambers every hour. Each chamber contained five layers of wire cages (nine cages per layer), and each cage held 12 mice, for a total of 545 mice (50% males, 50% females). The aerosols were injected into the airstream at the top of the chamber and exhausted at the bottom. The animals were exposed for $5\frac{1}{2}$ hr daily for 5 days a week. The chemical treatments consisted of either artificial smog of ozonized gasoline or insoluble Cr_2O_3 dust. The layers of cages were rotated so that the weekly exposure was the same for all animals.

The artificial smog was created by evaporating a straight-run, unleaded gasoline into a stream of oxygen and ozone resulting from the exposure of pure oxygen to ultraviolet light. The chamber was continuously monitored for excess ozone and hydrocarbon; the maximum concentrations maintained were 1 ppm ozone and 24-30 ppm gasoline. The Cr_2O_3 was ground in a fluid energy mill and had an average bulk particle size by weight of 0.85 μ, as determined by a combination of micromerograph and electron micrograph techniques. The dust was dispensed into the inlet air by a Wright dust feed at a concentration of 25

mg/m^3. The dust was monitored by collection on Millipore filters from three levels in the chambers, and the chromium content determined chemically or by direct weighing of the Millipore discs.

Data Storage and Retrieval

Due to the complexity of maintaining manual records on the large number of experimental mice used in these inhalation experiments, and the laborious process involved in retrieving pertinent information from such records, six computerized experimental programs were developed. The main program created and periodically updated a master tape containing the experimental records of all animals used in the experiment. This master record is created through a series of control cards, plus data cards produced through a separate breeding phase computer program, and is partially updated with transactions made by an IBM 357 data transmission system. Pertinent information, listed in experimental cage order and sorted by chamber, includes the time of death and pathology record for each animal. Animal weights are recorded monthly. Both the survival and pathology records are sorted and analyzed under separate subprograms.

Statistical Analysis of Data

We considered differences associated with the main effects of the four variables (chemical, virus, sex, radiation) and their two-factor interactions (e.g., chemical × virus, etc.) as defined in the usual factorial design model.[10] As originally developed, the factorial design model provides for the simultaneous study of effects of combinations of different treatments [for a discussion of factorial experiments as applied to studies in inhalation carcinogenesis, see M. A. Kastenbaum and T. J. Mitchell, these *Proceedings*, p. 445]. In testing for differences among effects, the number of tumors in each group was assumed to be a Poisson random variable.[11] The measure of probability of significance was calculated for each difference, and a method was developed to select from the whole set of differences those which are "real" at the 0.05 level. Critical values for the test were calculated by using the first term of Bonferroni's inequality.[12]

Necropsy Procedure

Complete necropsy was performed on all animals except for those extensively cannibalized. All grossly visible pathological lesions were recorded on a necropsy record sheet. Sections were taken routinely from all major organs, except the brain, and were fixed in 10% neutral buffered formalin. The lungs were examined lobe by lobe and the number, size, and location of tumors was recorded. Histological sections were prepared from all fixed tissues and were routinely stained with hematoxylin and eosin. A minimum of three histological sections, 100–150 μ apart, were obtained from lung and trachea in order to increase the chance of identifying small lung tumors not visible on the pleural

surface. Histological and final diagnosis was recorded on the same record sheet used for the necropsy data.

RESULTS

The lung tumors observed in this study were pulmonary adenomas and adenocarcinomas, the latter comprising about 10% of the total. Since most of these tumors develop slowly, they are detected only when the animals die from some other disease. It is therefore important to determine the median survival time of the animals in the various experimental groups (that is, the time at which 50% of the total population has died), because a change in this parameter can also affect the lung tumor incidence. The median life-span of animals living in inhalation chambers (regardless of type of inhalation exposure) was 102.4 weeks, whereas that of mice living in ordinary mouse rooms within the barrier facility was 127.5 weeks (Fig. 1). Among the causative factors in this 25 weeks of life-shortening, the most important one may be that the chamber animals lived in wire cages; dermatitis and urogenital disease were the major causes of death in these animals. Among the nonchamber animals, all of which lived in regular mouse pans with bedding, dermatitis and urogenital disease were only occasionally observed.

Figure 2 shows that, independent of treatment, an increase in median survival time resulted in an increase in the cumulative incidence of lung tumors, indicating that lung tumor development is a function of age. The cumulative lung tumor incidence percent with time was plotted for two of the short-lived

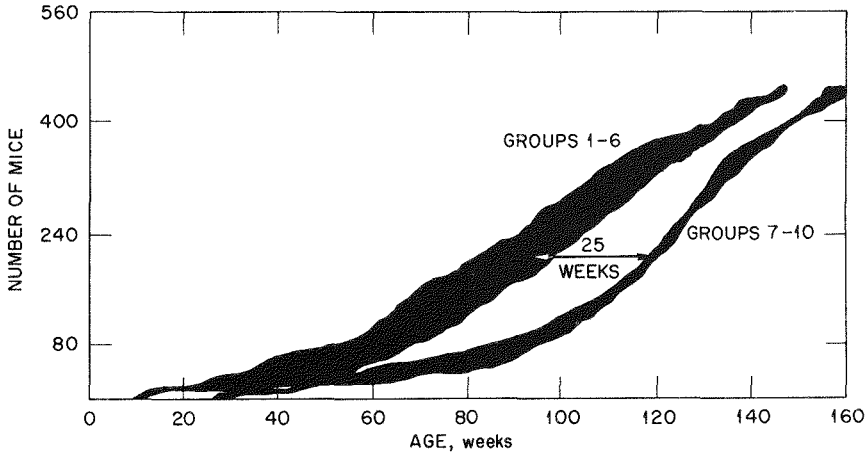

Fig. 1 – Cumulative mortality in all animals excluding those removed for testing. Groups 1–6 lived in wire cages and were put into the inhalation chambers 5 days a week. Groups 7–10 lived in regular mouse cages with bedding and were not placed in the inhalation chambers.

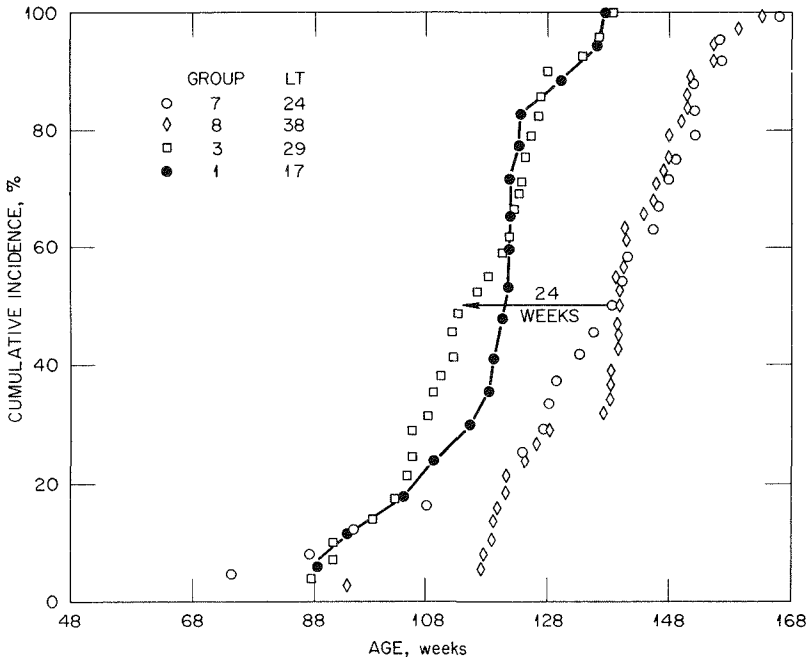

Fig. 2 – Cumulative incidence of lung tumors (percentages) in two groups of the nonchamber experiment (no treatment and 100 R only) and in two groups of the inhalation chamber experiment (filtered air and smog). LT = total number of lung tumors observed.

groups from the washed air and smog chambers (Groups 1 and 3), and for two of the long-lived mouse populations from the no treatment and 100 R only groups (Groups 7 and 8). Since the lung tumors are detected only if the animals die from some other cause, the tumor incidence curves for the long-lived animals are displaced for approximately 6 months, coinciding with the 6-months increase in median survival time of these animals. With the extension of life-span, the total number of tumors also increases (62 as compared to 46).

The total number of lung tumors obtained in the various groups and subgroups is summarized in Table 2. A comparison of the tumor yield in the two experiments reveals a tumor incidence of 5.5% in the nonchamber groups (124 out of 2200 animals) as compared to 3% in the chamber groups (99 out of 3300 animals). This reflects the approximately 6-months difference in life-span of the animals involved in these two studies. The data also show that sex has an effect on lung tumor incidence. In both experiments males developed more tumors than females (60♂ *vs.* 39♀ in the chamber experiment; 81♂ *vs.* 43♀ in the nonchamber experiment). Examination of the effect of pre-exposure to 100 R of whole-body X-radiation, regardless of any subsequent treatment, shows that irradiation increased the number of lung tumors in the females in both

TABLE 2

Lung Tumor Incidence

A. Inhalation chamber experiment

		Male			Female				
		X-rays	No X-rays	Subtotal	X-rays	No X-rays	Subtotal	Total	
No Virus	Air	7	3	10	5	2	7	17	61
	Cr_2O_3	2	7	9	6	0	6	15	
	Smog	11	13	24	4	1	5	29	
Virus	Air	5	0	5	2	0	2	7	38
	Cr_2O_3	2	3	5	2	5	7	12	
	Smog	3	4	7	10	2	12	19	
	Total	30	30	60	29	10	39	99	

B. Nonchamber experiment

No Virus			16	16		8	8	24	62
		19		19	19		19	38	
Virus			20	20		5	5	25	62
		26		26	11		11	37	
	Total	45	36	81	30	13	43	124	

experiments (29 *vs.* 10 in the chamber experiment and 30 *vs.* 13 in the nonchamber experiment), but had little if any effect on the lung tumor incidence in males (30 *vs.* 30 in the chamber experiment and 45 *vs.* 36 in the nonchamber experiment). In contrast, influenza virus infection suppressed lung tumor development in the mouse population involved in the inhalation study (38 *vs.* 61). However, this suppressive effect of influenza virus was not apparent in the nonchamber animals, which had a median life-span of 127 weeks (nonchamber experiment, 62 *vs.* 62).

In two inhalation chambers mice were chronically exposed to ozonized gasoline fumes. Half of each chamber population had been irradiated with 100 R of whole-body X-rays, and in one of the two chambers all animals had undergone viral pneumonia. In both groups of mice, smog raised the lung tumor incidence. In a similar experiment in which Cr_2O_3 dust was used instead of artificial smog, no increase in lung tumor incidence was observed.

In Tables 3, 4, and 5, the effects of the various agents are more closely examined, with differences in mean survival time taken into account. Since no interactions between pretreatments and chronic treatments were observed with any of the studied combinations, it is legitimate to consider each treatment by itself, regardless of any other treatment or pretreatment that the animals may have received. This increases the number of animals for each particular treatment considerably and thereby the chance of finding statistically significant effects.

Table 3 shows the frequency of lung tumors in both sexes and the effect of X-irradiation on lung tumor incidence. It can be seen in both experiments that in the unirradiated population, males developed three times more lung tumors than

TABLE 3

*Lung Tumor Incidence and Median Survival Time (Weeks) in Mice Exposed to X-Rays (Total 5500 Animals)**

Chamber experiment			Nonchamber experiment		
Sex	X-rays	No X-rays	Sex	X-rays	No X-rays
♀	29 (115.2)	10 (100.4)	♀	30 (118.6)	13 (120.6)
♂	30 (94.6)	30 (99.2)	♂	45 (134.6)	36 (136.3)
Total	59	40		75	49

*The effect of X-rays on females, as analyzed by a *factorial analysis,* is significant at the .05 level.

TABLE 4

*Lung Tumor Incidence and Median Survival Time (Weeks) in Mice Exposed to PR8 Virus (Total 5500 Animals)**

Chamber experiment			Nonchamber experiment		
Sex	Virus	No virus	Sex	Virus	No virus
♀	21 (108.9)	18 (106.7)	♀	16 (120.6)	27 (118.6)
♂	17 (91.9)	43 (101.9)	♂	46 (134.9)	35 (136.0)
Total	38	61		62	62

*The effect of virus on males in the chamber experiment, as analyzed by a *factorial analysis,* is significant at the .05 level.

TABLE 5

*Lung Tumor Incidence and Median Survival Time (Weeks) in Mice Exposed to Smog (Total 2200 Animals)**

Sex	Smog	No smog
♀	17 (114.9)	9 (112.3)
♂	31 (102.2)	15 (93.5)
Total	48	24

*The smog effect as analyzed by a *factorial analysis* is significant at the .05 level.

females (30 *vs.* 10 and 36 *vs.* 13). This higher lung tumor incidence in males cannot be explained on the basis of differences in life-span between the two sexes, since such a difference was seen only in the nonchamber experiment. X-irradiation raised the lung tumor incidence by approximately 50% in both

experiments (59 *vs.* 40 and 75 *vs.* 49). This was almost exclusively due to a marked increase in occurrence of lung tumors in irradiated females, since X-irradiation had little or no effect on tumor incidence in males. The increased lung tumor incidence could have been a consequence of the prolonged life-span of the irradiated females in the chamber experiment. However, the same X-ray-induced increase was also observed in the nonchamber experiment, in which the survival times of irradiated and unirradiated females were similar.

In the inhalation experiments, a comparison of lung tumor incidence between groups of mice pre-exposed to influenza virus and those not receiving this pretreatment reveals that virus infection decreased the frequency of lung tumors (Table 4). This suppression of lung tumor incidence occurred only in males. The biological significance of this finding is somewhat questionable, however, since the males receiving virus had a shortened median survival time. Also, no inhibitory effect of virus treatment was apparent in the nonchamber experiment. The effect may therefore represent a retardation of the tumor growth rather than a suppression of tumor induction.

Animals chronically exposed to ozonized gasoline fumes (Table 5) developed twice as many lung tumors as animals exposed to filtered air. This effect was observed in both males and females. Despite the suppressive effect of influenza virus on lung tumor incidence, the smog effect was not abolished in infected mice. Although the survival time of smog-exposed males was 9 weeks longer than that of nonexposed males, no such difference was seen between exposed and nonexposed females. The fact that the lung tumors actually occurred earlier in the smog chamber animals than in the filtered air controls (see Fig. 2) also indicates that the smog effect is not a consequence of extended life-span. We can therefore safely conclude that chronic exposure to ozonized gasoline fumes significantly increases the lung tumor incidence in mice.

A detailed account of the type and frequency of pulmonary and extrapulmonary pathological lesions found in our study will be given elsewhere. For the purpose of the present report, it may suffice to say that no pneumonitis was found in mice other than those purposely infected with PR8 influenza virus. The morphological lesion persisting after influenza virus infection was not appreciably modified by smog exposure. No squamous metaplasia was seen in conducting airways or lung parenchyme of any animals. The only lung tumors found were pulmonary adenomas and adenocarcinomas. The major nonpulmonary, often fatal lesions were: chronic dermatitis, urogenital disease, lymphoma and reticulum cell sarcoma, severe glomerulosclerosis, and ovarian tumors.

The main findings can be summarized as follows: In C57BL/6 mice, males had a significantly higher incidence of lung tumors than females; irradiation raised the incidence of lung tumors in females but not in males; chronic exposure to artificial smog increased the lung tumor incidence in both sexes; infection with PR8 influenza virus appeared to retard lung tumor growth; exposure to Cr_2O_3 dust had no discernible effect on lung tumor incidence.

Under the present experimental conditions, the combination of treatments had no additive or potentiating effects.

DISCUSSION

Induction of lung tumors in mice has proven to be a very sensitive assay system for testing the carcinogenic activity of a great number of agents (e.g., ref. 13). This animal model therefore lends itself to test even rather weak carcinogens and cocarcinogens. Thus Kotin and co-workers[14,15] reported an increase in lung tumor incidence in strain A and C57BL/6 mice exposed to artificial smog, and Gardner[3] found a slight increase in lung tumors of mice exposed to Los Angeles ambient atmosphere. Our data confirm these earlier findings that oxidation products of gasoline vapors are tumorigenic for the lung. The fact that the relationship between pulmonary tumors in mice and the commonly observed squamous cell tumors in man is problematic does not invalidate this conclusion.

Previous experiments by Campbell[16] and by Steiner and Loosli[17] suggested that influenza virus infection may be inhibitory to chemical induction of lung tumors in mice. Kotin's[2] data on effects of multiple virus treatment and smog exposure on C57BL/6 mice also suggest inhibition of lung adenoma formation by influenza virus. The results obtained in our own study confirm these observations and suggest that influenza virus infection in some still undefined manner retards lung tumor growth in mice.

There is a major difference between our findings and those reported earlier by Kotin and associates.[1,2,13] It is related to the morphological changes observed after smog and virus-plus-smog exposure, and may be of great significance. These authors reported pneumonitis and epithelial hyperplasia with squamous metaplastic changes in their control animals; the incidence of the latter was greatly increased by smog exposure. Smog exposure also caused what these workers termed "smog pneumonitis." In their study mice were repeatedly infected with influenza virus and murine pneumonitis virus while being exposed to ozonized gasoline, and squamous cell tumors subsequently developed. In our experiment neither spontaneous pneumonitis, smog pneumonitis, squamous metaplastic changes, nor squamous cell tumors were encountered. The multiple virus treatment in Kotin's experiment seems to be the only major difference in experimental design; otherwise, it seems that the similarities far outweigh the dissimilarities. However, the mice used in his studies apparently had a high spontaneous incidence of pneumonitis.

We believe that the difference in microbiological status between our animals and those used in Kotin's investigations may be the major reason for the differences in histopathological findings. We therefore feel that the results of the previous studies and our findings are not contradictory, but rather complimentary. Taken together they very strongly suggest that infectious agents,

whether adventitious or purposely introduced, may be instrumental in determining the morphological and biological characteristics of lung tumors induced by chemical carcinogens.

ACKNOWLEDGMENTS

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REFERENCES

1. P. Kotin and D. V. Wiseley, Production of lung cancer in mice by inhalation exposure to influenza virus and aerosols of hydrocarbons. In, *Progress in Experimental Tumor Research,* Vol. 3, pp. 196–215. Karger, Basel/New York, 1963.
2. P. Kotin, The influence of pathogenic viruses on cancers induced by inhalation. In, *Canadian Cancer Conference,* Vol. 6, pp. 475–498. Pergamon Press, New York, 1966.
3. M. B. Gardner, Biological effects of urban air pollution. *Arch. Environ. Health,* 12 (1966) 305–313.
4. A. M. Baetjer, Pulmonary carcinoma in chromate workers: I. Review of literature and report of cases. *Arch. Ind. Health Occupational Med. Hyg.,* 2 (1950) 487–504.
5. W. C. Hueper and W. W. Payne, Experimental studies in metal carcinogenesis: chromium, nickel, iron, arsenic. *Arch. Environ. Health,* 5 (1962) 445–462.
6. C. H. Steffee and A. M. Baetjer, Histopathologic effects of chromate chemicals. *Arch. Environ. Health,* 11 (1965) 66–75.
7. E. Lorenz, J. W. Hollicraft, E. Miller, C. C. Congdon, and R. Schweisthal, Long-term effects of acute and chronic irradiation in mice. I. Survival and tumor incidence following chronic irradiation of 0.11 r per day. *J. Natl. Cancer Inst.,* 15 (1955) 1049–1058.
8. E. Lorenz, Some biological effects of long continued irradiation. *Am. J. Roentgenol. Radium Ther. Nucl. Med.,* 63 (1950) 176–185.
9. M. L. Simmons, C. B. Richter, J. A. Franklin, and R. W. Tennant, Prevention of infectious diseases in experimental mice. *Proc. Soc. Exptl. Biol. Med.,* 126 (1957) 830–837.
10. G. E. P. Box and J. S. Hunter, The 2^{K-p} fractional factorial designs, part I. *Technometrics,* 3 (1961) 311–351.
11. A. Birnbaum, Statistical methods for Poisson processes and experimental populations. *J. Am. Statist. Assoc.,* 49 (1954) 254–266.
12. W. Feller, *An Introduction to Probability Theory and its Applications.* John Wiley & Sons, Inc., New York, 1957.
13. M. B. Shimkin, Pulmonary tumors in experimental animals. In, *Advances in Cancer Research,* Vol. 3, pp. 223–267, J. P. Grundstein and A. Haddow (eds.). Academic Press, New York, 1955.
14. P. Kotin and H. L. Falk, II. The experimental induction of pulmonary tumors in strain A mice after their exposure to an atmosphere of ozonized gasoline. *Cancer,* 9 (1956) 910–917.

15 P Kotin, H L Falk, and Ch J McCammon, III The experimental induction of pulmonary tumors and changes in the respiratory epithelium in C57BL mice following their exposure to an atmosphere of ozonized gasoline *Cancer,* 11 (1958) 473–481
16 J A Campbell, Influenza virus and the incidence of primary lung tumors in mice *Lancet,* 2 (1940) 487
17 P E Steiner and C G Loosli, The effect of human influenza virus (type A) on the incidence of lung tumors in mice *Cancer Res* 10 (1950) 385–392

DISCUSSION

O. G. Raabe: I have two related questions First, I would like to know something about your chromate aerosols What particle size distribution were you working with, and how did you generate them?

The other question concerns the artificial smog What were the particulate concentrations and the vapor concentrations in this material?

P. Nettesheim: The chromium oxide dust was generated by a Wright dust feed. I wonder, for the purpose of clarity and more information, whether Dave Doherty or Ray Newell would like to give any more information that we have on the dust, besides the data that I mentioned?

D. G. Doherty: The chromium oxide dust was supplied by a Wright dust feed, as Paul mentioned, and the chamber concentration was 50 ppm The dust is a very dry, friable powder with no electrostatic charge and therefore no aggregation The particles were collected in the chamber by various means – slides, filters, and electron microscope grids – and it was found that in the size delivered, 90% of the particles were between 0 5 and 1 5 μ

By count, there was a large number of very small particles, approximately 10% by weight were 0 2 μ or below.

Gasoline was evaporated into the stream of inlet air by use of a heater around the inlet duct, it also went through the ozone tube, and was irradiated with UV and ozonized at the same time We maintained 1 ppm of excess ozone and monitored the hydrocarbon content continuously The smog was delivered as a vapor, and the concentration was maintained between 23 and 30 ppm

F. J. C. Roe: I wasn't quite sure about the disease-free status of your animals

Nettesheim: What I said was that germfree-derived animals were used, in other words, the animals were derived from a germfree colony We conventionalized them by administering four different strains of bacteria – I couldn't tell you right now what these were. The mice were kept in a very strict barrier and were continuously tested They remained free of the seven mouse viruses that we could test for, and they remained free of any bacterial pathogens

Roe: Thank you. If you were doing this experiment again, would you still choose a strain of mouse with a low incidence of lung tumors, or would you choose one with a higher incidence?

Nettesheim: I would rather not have to make that decision any more! But perhaps I can comment a little more sensibly: I think we are dealing with a problem here. If you have a low-incidence tumor strain, you are probably also faced with a very tough problem as far as chemical induction is concerned. But, I think, if you are dealing with such a mouse, every tumor that you get is much more meaningful, and probably also weighs more heavily statistically, than in a strain having a high spontaneous incidence, like 30 or 50%. In these strains you are not sure whether you are just enhancing the development of lung tumors a little or are actually inducing lung tumors.

On the other hand, I agree that if you were working with a strain with a higher spontaneous lung tumor incidence, you might not have such a hard time getting an increase in lung tumors, so that would be the benefit.

Roe: May I suggest that there are a number of other parameters which are useful in the case of high lung tumors: for instance, multiplicity of tumors, size of largest tumors, and histological grade of malignancy.

Nettesheim: Yes, except for the first one that you mentioned, namely, multiplicity of tumors, which we encountered only three or four times, I believe. All the other parameters are available to us, too. Of the tumors we observed, 50% involved one of the smaller bronchi. The histological characteristics of most of these tumors were that they were rather papillary, and not the more solid tumor that you see typically arising in the alveolar areas, separate from any connection with the bronchus. We did have some tumors metastasizing into other lobes. We also had some tumors that spread throughout the whole lung.

H. G. Petering: I was interested in the effect of PR8 virus on cancer induction. We know that there is some evidence that carcinogens depress immune response while viral infection may stimulate it. Have you considered the interrelationship here? Do you know anything about the immune status of your animals and the effects of treatment on it?

Nettesheim: We have some speculations, if you care to have them. Whether they are worth anything is a different matter. Dr. Loosli saw a suppression of lung adenoma induction by influenza virus infection in his experiments; and, as I mentioned, if you look at the data in Paul Kotin's experiments, you find this suppression there, too. Dr. J. A. Campbell, in earlier studies [*Lancet,* 2 (1940) 487], also saw it. So I think it is real. In our own experiments the suppressive effect of influenza virus on lung tumor incidence was seen in the shorter-lived mouse population but disappeared in the animals with an extended life-span. This suggests that the suppressive effect was only a temporary one that retarded tumor growth, rather than one that interfered with tumor induction.

So I think there are several possibilities here that one could think of. Maybe the virologists present will object to my speculation; but it could be, for example, that interferon has something to do with the inhibition of tumor

appearance. There is some evidence that some of these lung tumors are viral in origin. Not only have various people described viral particles, but also there is some evidence in the German literature [P. Klärner and Ursula Westphal, *Experientia,* 21 (1965) 435; P. Klärner and Brigitte Albrecht, *Naturwissenschaften,* 53 (1966) 44] that the tumors can be induced with cell-free extracts from pulmonary adenomas. So if one thinks in terms of a viral etiology, the possibility might exist that the influenza virus infection would stimulate interferon, and thereby at least temporarily retard either induction or growth of the tumor. That's one possibility.

The other thing that is very striking for the morphologists, and I am not implying anything beyond that, is that the PR8 influenza virus lesions which persist in most animals throughout their lifetimes – we used to call them pseudoglandular transformations of the alveoli – are very similar morphologically in many respects to some of the pulmonary adenomas.

Now, I don't know, as I say, whether this is simply morphology. I hesitate even to mention it, but it could be that the adenoma tissue and the virus-induced metaplasia have similar antigenic components and that immunization occurs in those animals and might protect them from the adenoma formation. This is, you know, purely speculation, but I think it might be worthwhile to look into it more closely.

C. G. Loosli: Inasmuch as some of our studies have been referred to, I think it might be appropriate to comment. Before I do so, I would like to comment on Dr. M. B. Gardner's studies, which were extended since his first report in 1963 to include 7000 mice, divided into two groups – the C57BL tumor-resistant strain and the tumor-susceptible AJ strain.

As I recall there was no difference in incidence of tumors between the tumor-resistant strain of mice living out their lifetimes in the ambient Los Angeles air and those living in the filtered room air. However, there were fewer lung adenomas in the tumor-susceptible mice living in the ambient air versus those living in the filtered air. These were not pathogen-free mice, however. Thus, there was a significant amount of pneumonitis in both groups of mice living in the ambient air compared to the filtered air group; in other words, those living in the clean-air environment had much less nonspecific pneumonitis than did those living in the ambient air. This was also the situation with several thousand rats that were observed over several years' study, namely, that the incidence of pneumonitis in the ambient air group was significantly greater than in the clean-air group.

With respect to our studies on the effect of air pollutants on influenza virus infections, we have been particularly concerned with the effect of NO_2 on the course of influenza virus infection in pathogen-free mice. We use the aerosol technique of inoculation and quantitate the infections carefully. Under these circumstances we can show that NO_2 inhalation conditions the animal to become significantly more resistant to influenza virus infection than is the

animal not previously exposed to the NO_2 atmosphere. At first we thought this was due to the lung lesions provoked by NO_2. However, we subsequently exposed different groups of animals to several concentrations (2, 5, 10, 20, and 35 ppm) of NO_2 for varying periods of time and found that all groups were significantly more resistant to influenza virus infections that killed 90% of the mice not exposed to NO_2.

We also found that this condition persisted, even after the animals were removed from NO_2 chambers for as long as 2 weeks. Something appears to be conferred on these animals which rendered them more resistant to airborne influenza PR8-A virus infection. Our studies to date indicate that this represents an increased interferon response with NO_2-exposed mice. The lungs of animals exposed to the NO_2 atmosphere compared to those living in ambient air show a much greater quantity of interferon after exposure to the influenza virus. This can be demonstrated in the lung tissue of animals killed 24 and 48 hr after onset of infection. The mechanism of action of interferon to bring about increased survival of the NO_2-exposed mice is not yet clear. As far as we can determine, the growth of the virus is essentially the same in the NO_2-exposed mice as in the control groups. In the lungs of the NO_2 groups the virus grows somewhat more slowly, but by 3 days the virus titers in the lungs are about the same in both groups. Likewise, the antibody responses in the NO_2-exposed and control animals recovering from influenza infections after 10 or 12 days are the same.

STUDIES IN PULMONARY CARCINOGENESIS

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ABSTRACT

Epidemiological and experimental evidence has increasingly indicated that the interplay of multiple environmental factors is responsible for the induction of lung cancer. Man is exposed to a complex mixture of potentially hazardous materials, including specific carcinogens and a variety of agents which may modify the manner in which the lung handles inhaled materials or responds to them. This laboratory has been engaged in a program to isolate and identify these environmental factors and to evaluate their effects. — Bronchogenic carcinoma, histologically similar to that seen in man, has been successfully produced in high incidences in this program. The techniques involve inhalation exposures with rats, surgically implanted intrabronchial pellets in rats and hamsters, surgically implanted threads in rats and mice, and intratracheal intubations in hamsters. Dose-response relationships for the induction of squamous cell carcinomas have been developed from the intrabronchial pellet technique with both polycyclic hydrocarbons and ionizing radiation. — Compounds of chromium have, for the first time, been demonstrated to be capable of producing bronchogenic carcinoma of the lungs in experimental animals. Of a special interest in this study is the production of both squamous cell carcinoma and adenocarcinomas correlating with human findings. — Inhalation of benzo[*a*]pyrene in combination with exposure to sulfur dioxide has resulted in the production of squamous cell carcinomas. These findings are of particular significance since both materials have been implicated in community air pollution problems and since these represent the first duplication of human lung cancer in animals by inhalation exposures to polycyclic hydrocarbons.

Our objective in these studies was to develop an animal model system which could duplicate the predominant carcinoma found in man. While we recognized the existence of spontaneous tumors and the variety of early metaplastic changes, our specific target was the development of true squamous cell carcinoma of bronchial origin. Although many of the early metaplastic changes are suggestive and certainly exciting, and of extreme importance in the

pathogenesis of the disease, our classification of carcinoma would accept only those findings that were definitely invasive and met all of the acceptable histological criteria. The point of view adopted, therefore, was rather conservative and pathologic evaluation severely limited.

The investigations followed two parallel directions. One was the introduction of large quantities of material via surgical implants or intratracheal instillations and the second was the development of an inhalation exposure system in order to more closely duplicate the exposures of man. Since these investigations involved a long series of studies extending over a period of approximately 18 years, this summary, at best, will only be able to discuss a few selected topics. In our early studies,[1] we performed a number of inhalation exposures with a variety of carcinogens. With 3-methylcholanthrene (MCH), these included exposures to pure fume, saline suspensions, and solutions in oil droplets and solid parafin aerosols. Concentrations ranged from 0.1 mg/m^3 up to 42 mg/m^3. In spite of exposure periods varying from 76 to 260 days of repeat exposures and prolonged observation periods, no lung tumors comparable to the human tumor were produced. Adenomas and skin tumors, however, were seen in animals exposed to MCH dissolved in corn oil.

Intratracheal injection of pure carcinogens was extensively investigated in both rats and mice. In these studies, the trachea was exposed and the material under test slowly injected into the lumen. With this technique, only adenomas and paratracheal sarcomas were produced. The data suggested an increase in the incidence of these tumors with dose, as well as differences in strain susceptibility. Several squamous cell carcinomas of tracheal origin, however, were produced as a result of accidents in performance of the technique. In the accidents, material was deposited in the submucosal tissue during the course of injection. The finding of squamous cell carcinomas in these situations demonstrated that if enough material was present at the target area, true cancer could develop.

Studies of the rate of disappearance of carcinogen introduced by inhalation or intratracheal injections were performed by means of fluorescence microscopy and quantitative chemical determination on lung homogenates. These investigations showed an extremely rapid clearance of material that was essentially complete in 24 hr following intratracheal injection and in 6 hr following inhalation.

Subsequent studies involved the use of techniques in which the role of irritants, injury, and other co-factors were examined. One technique of considerable interest involved the use of carcinogen-impregnated threads surgically implanted in the lung. With a modification of the Andevont[2] technique in mice, high yields of squamous cell carcinoma were produced with dibenz[*a*,*h*]anthracene (DBA). Further modification of the technique for rats was made by the use of open thoracotomies and direct transfixation of the lung. This was extremely successful in producing cancer incidences in the order of 40% with materials such as benzo[*a*]pyrene (BP), DBA, and MCH. These tumors

were well-differentiated squamous cell carcinomas with a marked tendency to local invasion and metastases to the lymph nodes, pleura, and in several cases, extensive involvement of the kidneys.

Since we have previously described these techniques and their results,[1,3] just two phases of our current studies will be emphasized here: the intrabronchial pellet implant technique, and our more recent inhalation studies.

INTRABRONCHIAL PELLET IMPLANT TECHNIQUE

The pellet technique was devised to afford selective exposure of a portion of the bronchial mucosa to high concentrations of carcinogenic materials. Using anatomical models derived from vinyl and from Wood's metal casts, we fabricated pellets to fit the left inferior bronchus of rats. These pellets are in the form of a cylindrical matrix constructed of stainless steel wire mesh (Fig. 1). Each pellet is approximately 1 mm in diameter and 5 mm long. The pellets were impacted in the bronchus by means of a trochar introduced through a tracheotomy (Fig. 2). It was necessary to fit the pellets with spring wire hooks since the animals coughed up unhooked ones. The pellets were impregnated with polynuclear hydrocarbons by immersion in molten material. The amount of material contained by the pellet after immersion varied from 3 to 5 mg.

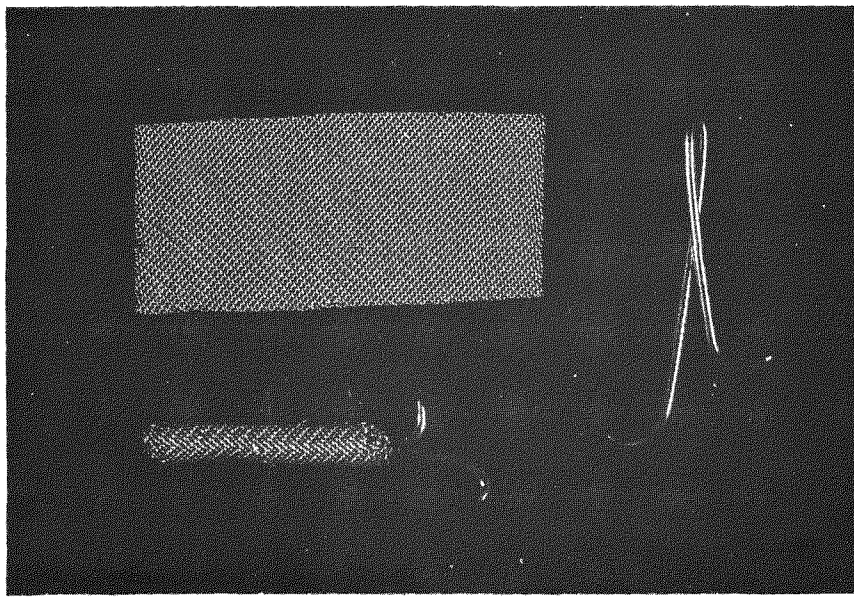

Fig. 1 – Stainless steel wire mesh, spring wire hooks, and assembled cylindrical pellet with hooks incorporated. (From Kuschner *et al.*[1])

Figure 3 shows an X-ray of a rat with the pellet in place Initial studies with BP, DBA, and MCH showed yields of squamous cell carcinomas ranging from 30 to 50% Table 1 demonstrates more recent data with animals exposed to MCH

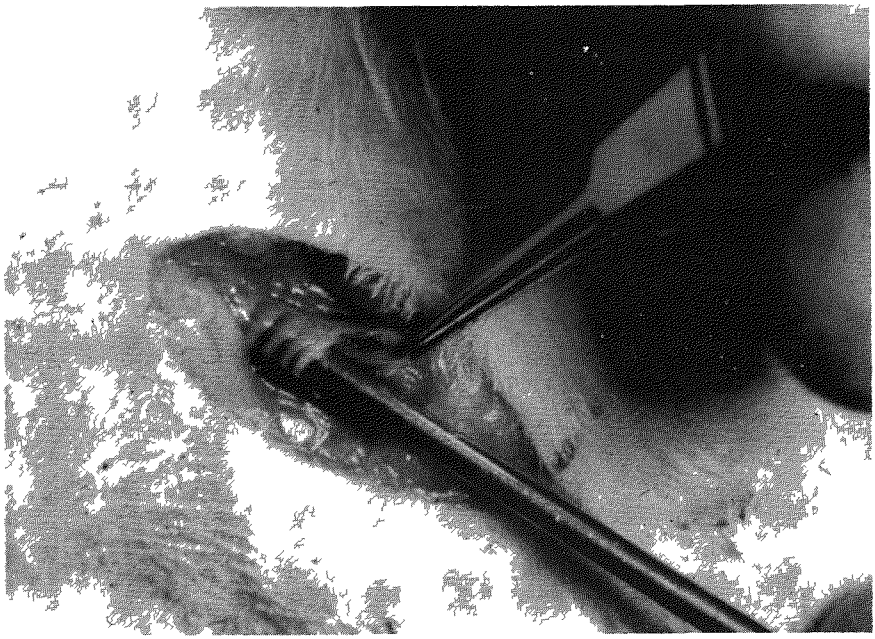

Fig 2 – Technique of pellet implantation in the rat bronchus The pellet in a trochar is introduced into a bronchus through a tracheotomy (From Kuschner *et al* [1])

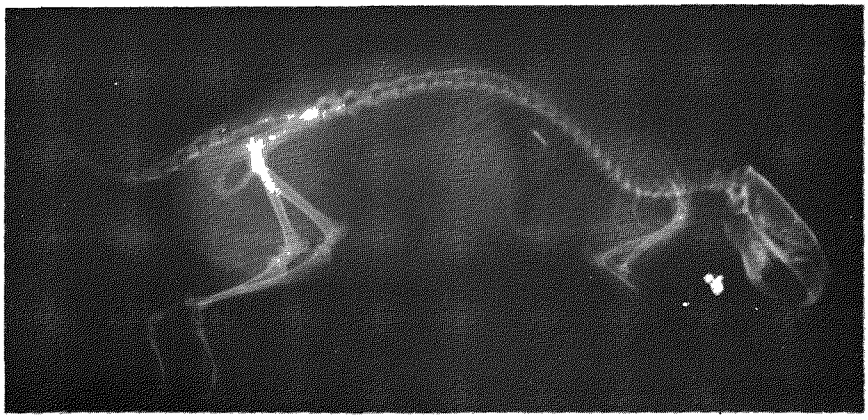

Fig 3 Roentgenogram of a rat with a pellet in place in the left main bronchus toward the base of the lung (From Kuschner *et al* [1])

In these studies, still incomplete, 100 animals in each group were exposed to pellets containing 3 mg of material ranging from 0.1 to 100% carcinogen. Lower concentrations were produced by dilution with cholesterol. The yield of squamous cell carcinoma was excellent, ranging from 37.9% down to 0 at the lowest level. The 186 controls, which contained cholesterol or blank pellets, showed no evidence of spontaneous carcinoma. Table 2 shows the major pathological changes observed with controls, MCH, and BP.

These findings represent an overall picture without regard to dose. The squamous cell carcinoma incidence was 20% with MCH and 11.6% with BP. Small amounts of undifferentiated carcinomas appeared with each carcinogen, and adenocarcinomas appeared only with MCH. Lymphomas and sarcomas

TABLE 1

Incidences of Squamous Cell Carcinoma in Rats Following Exposure to 3-Methylcholanthrene-Containing Pellet Implants

Pellet concentration (% MCH*)	No. of animals observed	No. of animals with carcinoma	Carcinoma incidence (%)
100	95	36	37.9
50	97	27	27.8
25	97	20	20.6
10	66	11	16.7
1	63	4	6.3
0.1	73	0	0.0
Controls	186	0	0.0

*Diluted with cholesterol.

TABLE 2

Major Pathological Changes Observed in Rats Following Exposure to Carcinogen-Containing Pellet Implants

Pathological changes	Percent of animals showing changes			
	Controls		Carcinogens	
	Wire mesh	Cholesterol	Methylcholanthrene	Benzpyrene
Squamous metaplasia	17.2	20.4	24.2	20.8
Squamous cell carcinoma	0.0	0.0	20.0	11.6
Undifferentiated carcinoma	0.0	0.0	0.4	0.2
Adenocarcinoma	0.0	0.0	0.2	0.0
Lymphoma	1.1	0.0	0.2	0.4
Sarcoma	5.4	1.1	2.9	2.7

without further classification occurred in higher percentages in the wire mesh controls. It is interesting that squamous metaplasia occurred in high incidences in both controls and carcinogen-treated groups. The incidence was somewhat higher with MCH, but the significance of this finding is doubtful. The high incidence of squamous metaplasia is a well-recognized phenomenon following exposure to irritants and infection and may be of significance in the development of cancer, but is beyond the scope of the present discussion.

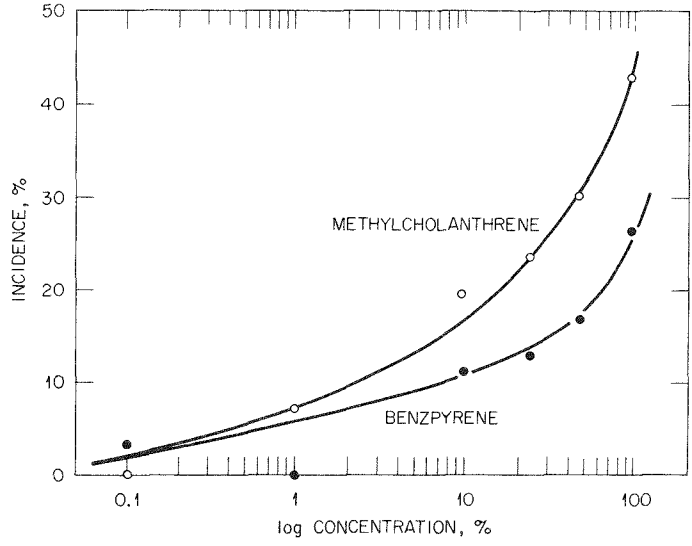

Fig. 4 – Dose-response relationship after exposure of the lungs of rats to a graded series of concentrations of 3-methylcholanthrene and benzo[*a*]pyrene on pellets. (From Kuschner[3])

Figure 4 summarizes the data with MCH and BP as a plot of incidences of carcinoma versus carcinogen concentration. These curves demonstrate a reasonably good dose-response relationship. Their classical appearance impressed us as being representative of the type of response observed with most drug reactions.[4] The data represented on these curves has been corrected for early experimental mortality. Estimates of maximum yields are 45% cancer incidence with MCH and 30% with BP. In order to make more adequate estimates, we plotted the data by the technique of probit analysis[5] (Fig. 5). This is exactly the procedure used for determining LD_{50}'s in which the log concentration in percent is plotted against the cancer incidence on log probability paper. The excellent straight lines obtained again suggest classical dose-response curves characteristic of the pharmacology literature. Interpretation of the data between the dosage points is valid, although extrapolation to lower values would require significantly larger groups of animals to provide statistical reliability.

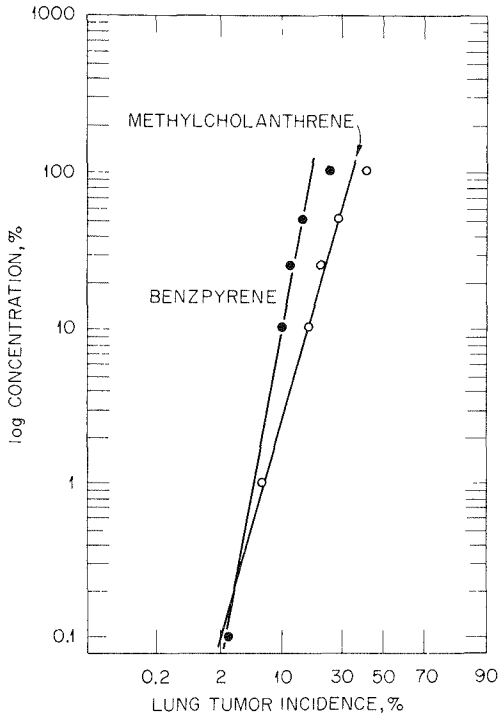

Fig. 5 – Log probability distribution of the relationship between dose of chemical carcinogens and squamous cell carcinoma of the lung in rats. (From Kuschner[3])

Implantation of Radioactive Pellets

Lung cancer of bronchogenic origin in rats and mice has been induced in several laboratories with alpha, beta, and gamma radiation. Inhalation, intratracheal injection or insufflation, and "blind" implantation within the lung have been used.[6-13] The calculated dose in these studies was generally expressed as an average lung dose or a mean dose within a sphere of irradiated tissue. Such interpretation was required because the spatial relationship between deposited or implanted material and the site of origin of the tumor could not be determined. Except with such highly penetrating radiation as the gamma source used by Gates and Warren,[13] this procedure is of questionable validity. Further, the tumor yields in most experiments were too low to permit the derivation of meaningful dose-response relationships.

The successful induction of squamous cell carcinoma with carcinogen-impregnated pellets suggested that the pellet technique might be the ideal one for exploring the problems of radiation-induced carcinogenesis. Figure 6 illustrates a modification of the pellet involving the use of a hollow platinum cylinder, 5 mm in length and 1.2 mm in diameter, with a wall thickness of 0.2

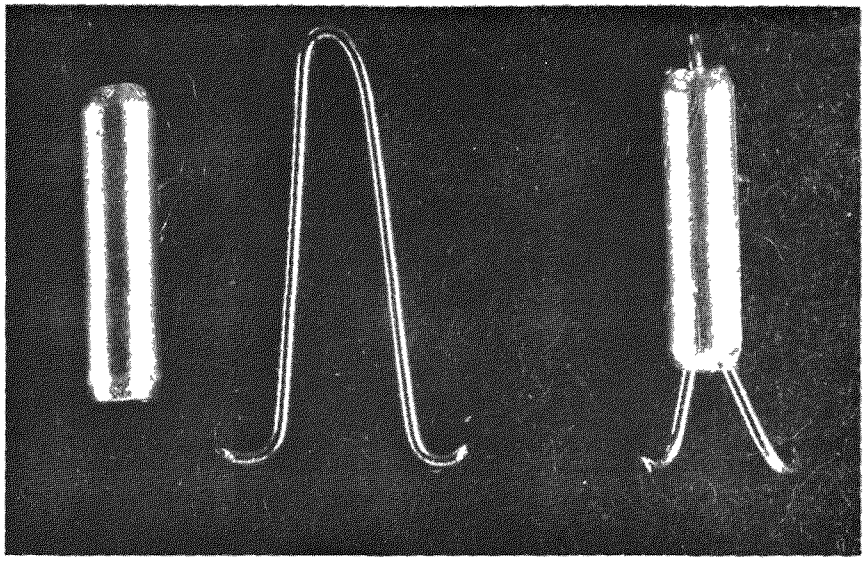

Fig. 6 – Spring wire hooks, platinum cylinder, and completed pellet with hooks incorporated. Pellet was plated with ^{106}Ru in rhodium carrier. (From Laskin *et al.*[15])

mm. The ^{106}ruthenium-rhodium system was selected for study because its emission was predominantly a 3.5 Mev beta with a half-life of 1 year. Since the system and its end product, palladium, were all noble metals, chemical effects could be avoided. The source material, ruthenium in rhodium carrier, was readily electroplated on the surface of the pellet. Plated pellets were protected against loss by a second plating of an equivalent amount of rhodium carrier.

The malignant tumors observed in these studies arose from the basal layer of the bronchial epithelium. Thus, the dose was calculated with this layer as the target tissue.[14] In the calculation the distance from the pellet to the target tissue was estimated at 100 μ. Errors in this estimate would not significantly alter dose calculations unless the actual distance was less than 25 μ, the maximum range of the soft beta particles derived from the decay of ^{106}Ru to ^{106}Rh.

Groups of rats were exposed to beta-radiation from ^{106}Ru-plated pellets implanted within the bronchi.[15] Microcurie levels varied from 0.008 to 13.6, and integrated dose from 10^3 to 10^6 rads. Since the spatial relationship of the emitting pellet to the bronchial origin of tumor was known, dose to the specific target tissue could be directly calculated. This is in contrast to previous quantitations of radiation to the bronchus in terms of "average lung dose."

Sixty-eight animals with squamous cell carcinoma were found with the 265 rats exposed. Figure 7 shows the incidence of cancer at various levels expressed in terms of microcurie dose. These results demonstrate a significantly higher incidence of cancer production than that induced with chemical carcinogens. A

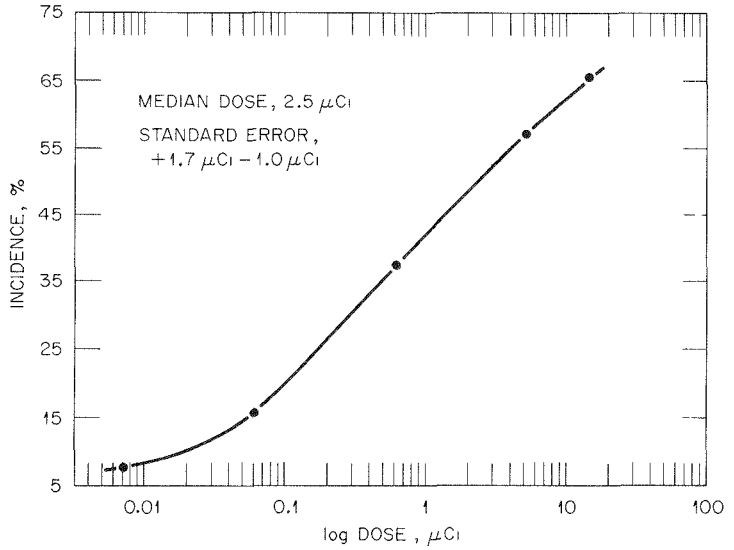

Fig. 7 – Dose-response relation in microcuries after exposure of the bronchial mucosa of rats to the β-radiation of ^{106}Ru-^{106}Rh pellet implants. Squamous cell carcinoma was found in survivors beyond 143 days. (From Laskin *et al.*[15])

more critical examination of the dose-response relationship was made from the study of tumor yields as a function of the integrated dose. Since the end point in this study was the death of the animals, variations in the time of death affected the accumulated dose in rads at each microcurie level. After regrouping the data we calculated tumor incidences for five orders of magnitude of rad dosage. Figure 8 shows the dose-response relationship obtained. The data is plotted on the log probability basis in Fig. 9. The excellent straight line obtained is again confirmation of the classic dose-response relationship of pharmacologists. The median dose was estimated at 3.02×10^5 rads. Standard errors and other statistical evaluations could be adequately made. In these studies, it was obvious that animals dying very early could not have contributed to the cancer incidence, and the data was therefore corrected. An analysis of the tumor-induction times showed a mean of 344 days with a standard error of 10.5 days. A cut-off point was calculated as 143 days, before which the probability of a tumor occurring was 1%.

An additional factor, which must be recognized in the calculation of dose, was that all calculations were based on the time of death with cancer, rather than the time of initiation of irreversible malignant change. Subsequent serial sacrifice studies demonstrated that this correction might be as little as 30 days. This "wasted radiation" is less than one-fifth of the total dose and makes only a minor contribution to the dose. In spite of the limitations of this study, a particularly striking outcome was the induction of three tumors in the 10^3 rad range; one of these was in an animal that had received 1400 rads. These results

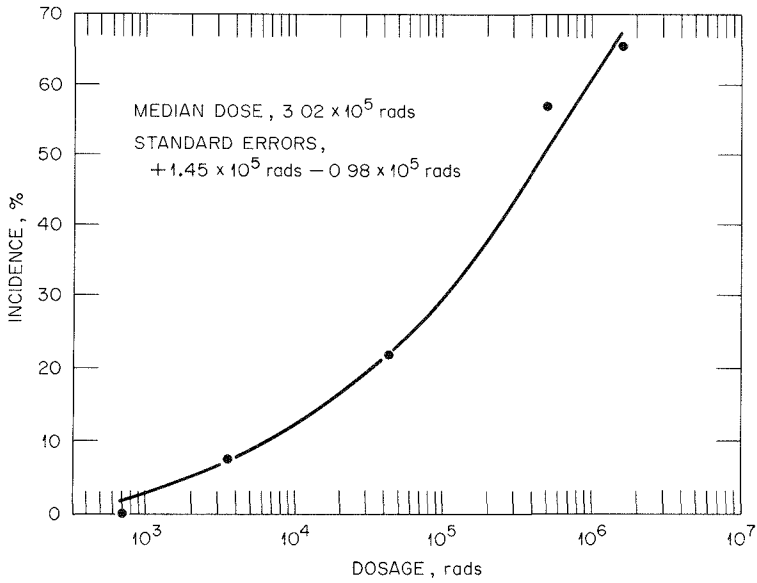

Fig 8 – Rad dose-response relationship after exposure of the bronchial mucosa of rats to the β radiation of ^{106}Ru-^{106}Rh pellet implants Squamous cell carcinomas in survivors beyond 143 days (From Laskin *et al* [15])

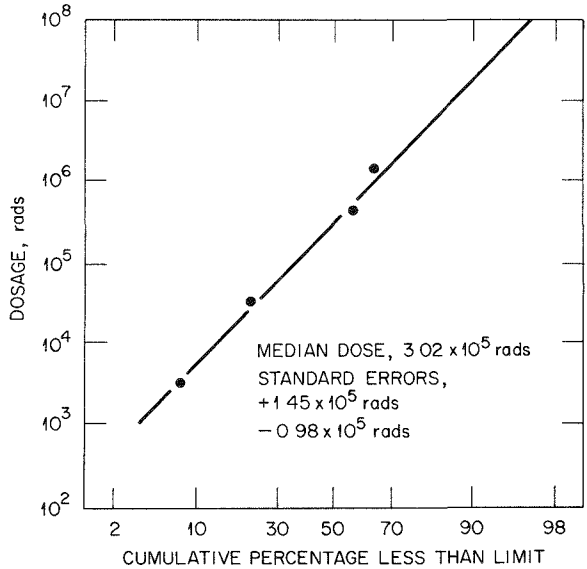

Fig 9 – Log probability distribution of dosage relationship between incidence of squamous cell carcinoma and radiation derived from a ^{106}Ru-coated pellet (From Laskin *et al* [15])

are comparable to the estimates of exposure levels and tumor incidence in Schneeberg and Joachimsthal[16] miners and, most recently, in uranium miners on the Colorado plateau.[17]

In order to critically evaluate the effect of such factors as dose rate and energy on radiation carcinogenesis, it was desirable to develop a procedure for utilizing isotopes of varying half-lives and beta energies. A hollow-core plastic pellet was devised which could be filled and sealed by a specially designed micropipetting apparatus. Such pellets permit the utilization of materials without regard to their physical and chemical properties. The completed pellet, illustrated in Fig. 10, is fabricated from an 8-mm length of polyethylene tubing with an inside diameter of 860 μ. Dosage uncertainty, arising from variation in the distance between the pellet and the mucosa, is minimized by the use of tubing with a wall thickness of 200 μ.

Studies were made with ^{32}P ($T_{1/2}$ = 14.3 days) in the form of soluble phosphate.[18] Groups of animals were exposed to pellets containing 20, 2, and 0.2 μCi. Dose estimates for these levels were 10^5, 10^4, and 10^3 rads, respectively. In these studies, incidences of squamous cell carcinomas were 10/18 at 10^5 rads, 5/15 at 10^4 rads, and 0/15 at 10^3 rads.

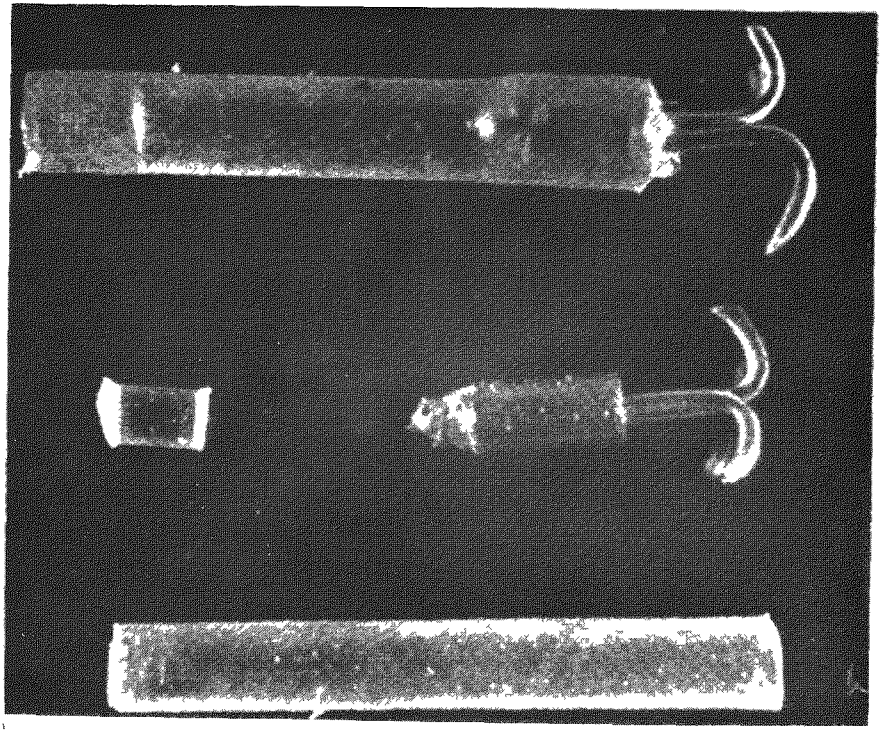

Fig. 10 – Plastic tubing, plug, and wire hook assembly combined to form complete hollow plastic pellet. (From Laskin *et al* [18])

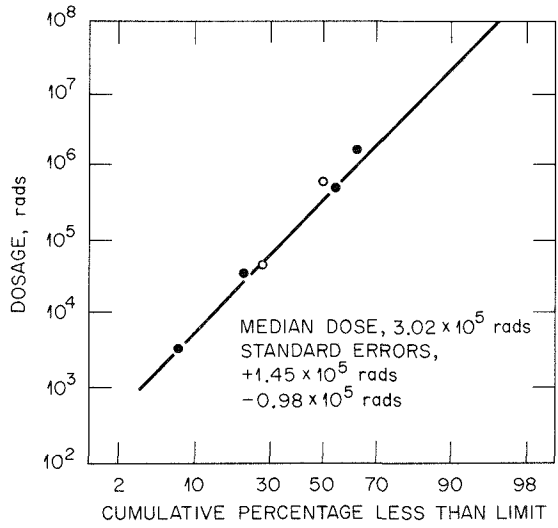

Fig. 11 – Dose-tumor response for ^{32}P plotted (open circles) on the log probability distribution for ^{106}Ru shown in Fig. 9. (From Kuschner[3])

Figure 11 shows the tumor response for ^{32}P plotted as open circles on the log probability distribution for ^{106}Ru shown in Fig. 9. The values of 56% at 10^5 rads and 33% at 10^4 rads show excellent agreement with the ruthenium data despite great differences in dose rates. Table 3 compares the cancer incidence obtained at 10^5 rads from two levels of ^{106}Ru and the comparable ^{32}P level. Where the dose rate has been determined by varying the amount of ^{106}Ru, or derived from shorter half-life ^{32}P, all tumor incidences are comparable at the same cumulative dose. Within the limits of these experiments, there has been no dose-rate dependence for tumor yield. This would appear to offer some justification for the use of "working level months" (i.e., the product of dose and time) as an estimate of human radiation exposure, since a large dose over a short time may be equivalent to a comparable dose accumulated at a lower rate over a more extended period.[17] Further studies are being developed to reinforce these observations on dose dependence.

TABLE 3

Duration of Radiation Exposure and Cancer Incidence with Bronchial Pellet Implants in Rats

Isotopes	Nominal activity (μCi)	Dosage range (rads)	Median survival time (days)	Median time to 75% dose (days)	Cancers	
					No./total	(Percent)
^{106}Ru	0.59	$1.2–2.2 \times 10^5$	462	302	11/18	61
^{106}Ru	5.0	$1.5–9.9 \times 10^5$	227	190	11/16	61
^{32}P	20.0	4×10^5	386	29	10/18	56

Implantation of Chromium Compounds

The chromium industry has been clearly associated with a significant incidence of human lung cancers. The annual respiratory cancer rate for male workers in the chromate industry in the United States has been reported as high as 29.4 times that of nonchromate workers of the same age distribution.[19] The distribution of neoplasias has been reported to be 79% squamous cell carcinomas and 21% adenocarcinomas.[20] Although this problem has been well recognized, no bronchogenic carcinomas of animals, simulating the human disease, have been reported in the literature.

Our successful application of the intrabronchial pellet technique led us to believe that it would be a valuable model for evaluation of this important industrial problem. A study of the literature, particularly Payne's important work in this area,[21,22] and an examination of the industrial processes suggested the materials shown in Table 4 as the most promising for investigation.

TABLE 4

Selected Chromium Compounds

Material	Formula	Valence	Relative solubility
Process residue			
(a) Chromates	CrO_4 =	6	Water soluble
(b) Chromate-chromite complex	CrO_4 = Cr_2O_4 =	3,6	Water insoluble; acid soluble
(c) Oxide	Cr_2O_3	6	Acid insoluble
Calcium chromate	$CaCrO_4$	6	Moderately soluble in water; soluble in alcohol
Chromic chromate	$Cr_2(CrO_4)_3$ $x(Cr_2O_3)y(CrO_3)$	3,6	? Soluble in water;* forms colloids
Chromic oxide	(x) Cr_2O_3	3	Insoluble in water and acids
Chromic trioxide	(y) CrO_3	6	Soluble in water, alcohol, ether, and acids

*CrO_3 added to freshly prepared $Cr(OH)_2$.

Studies of the carcinogenic effects of chromium compounds in a cholesterol carrier have been completed using the intrabronchial pellet technique. Compounds under investigation included chromic chromate, chromic oxide, chromic trioxide, calcium chromate, and process residue. Pellets were prepared from molten mixtures of materials dispersed in equal quantities of cholesterol carrier. These studies included materials of differing solubilities and valences, and have involved over 500 rats that were under observation for periods of up to 136 weeks.

Lung cancers that closely duplicate the human pathology were found in these studies (Table 5). With the calcium chromate, eight cancers were found in an exposed group of 100 animals. Six of these were squamous cell carcinomas found in animals dying from 386 to 671 days (Fig. 12). The mean cancer time for these findings was 540 days. One animal dying at 474 days showed metastasis to the kidney (Fig. 13). Two adenocarcinomas produced by calcium chromate were observed at 366 and 609 days (Fig. 14). Of special interest was a squamous cell carcinoma seen at 594 days in an animal exposed to chromate

TABLE 5

*Carcinomas Produced with Chromium Compounds in Rats**

Material	Number of animals	Squamous cell carcinoma	Adeno-carcinoma	Hepato-cell carcinoma
Process residue	100	1		1
Calcium chromate	100	6	2	
Chromic chromate	100			1
Chromic oxide	98			
Chromic trioxide	100			2
Cholesterol control	24			

*From Kuschner.[3]

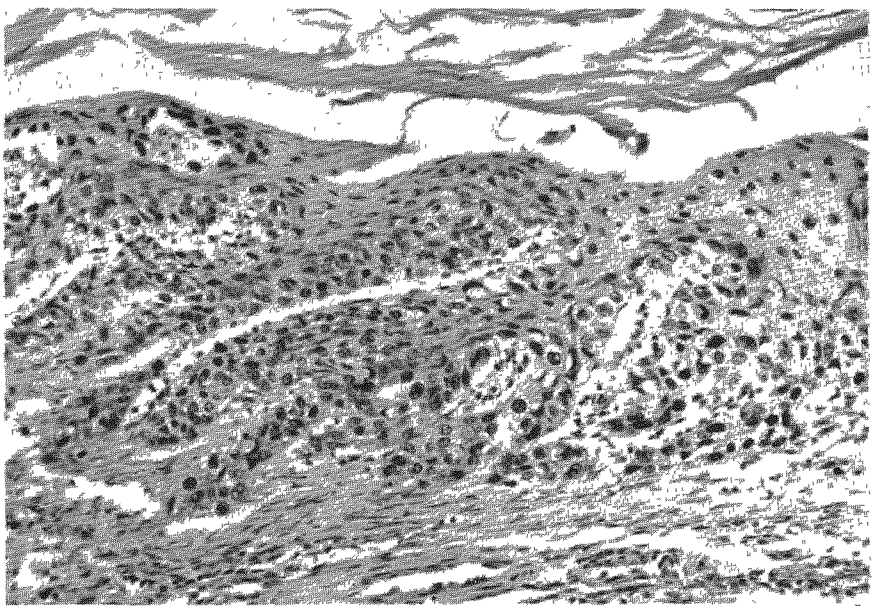

Fig. 12 – Squamous cell carcinoma arising in metaplastic epithelium in a rat exposed to calcium chromate. (265X; reduced 28%)

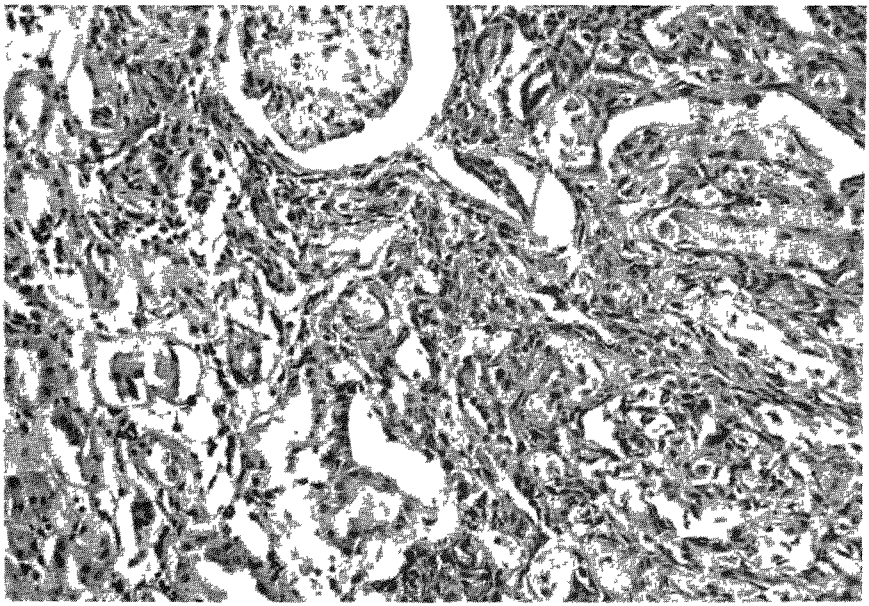

Fig. 13 – Secondary squamous cell carcinoma in kidney, surrounding a glomerulus and replacing tubular tissue. (265X; reduced 28%)

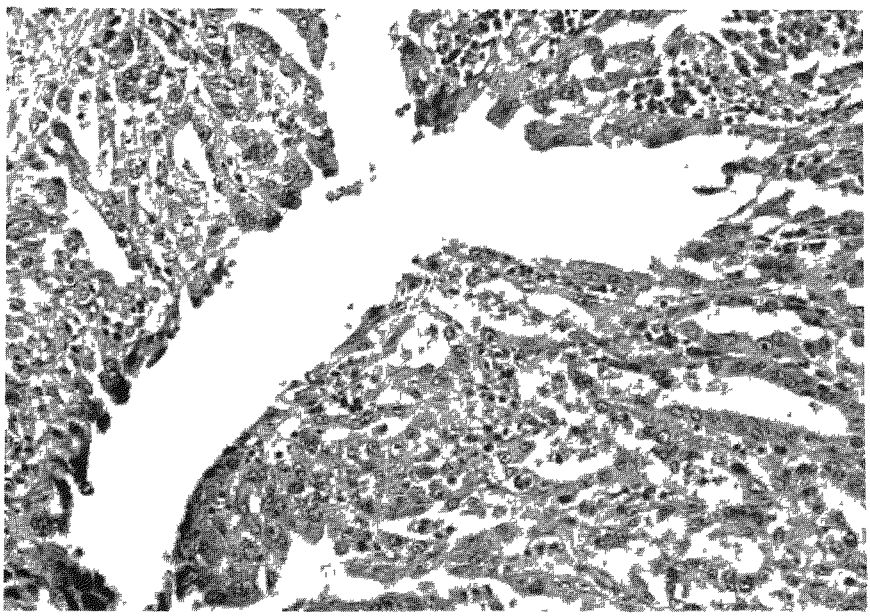

Fig. 14 – Adenocarcinoma completely replacing bronchial lining and extending periferally. (265X; reduced 28%)

process residue, an industrial intermediate containing soluble hexavalent components. Calcium may be present in the process residue to the extent of about 3%. In view of the fact that the material was diluted with cholesterol, it was remarkable to see any positive findings.

Additional findings of interest included four hepato-cellular carcinomas. One found at 873 days in an animal exposed to chromic chromate showed metastasis to the right lower lung. Two were found at 576 and 647 days in animals exposed to chromic trioxide, and one at 601 days in an animal exposed to process residue. With the exception of the animals exposed to chromic trioxide, all the experimental groups showed evidence of atypical squamous metaplasia of the bronchus.

Table 6 shows a comparison of carcinoma incidence with MCH, BP, and chromium compounds. All of the results were obtained with 1:1 mixtures of cholesterol and material and suggest a relationship between the potency of the carcinogen and cancer incidence. These range from 29.7% with MCH to 1.1% with process residue. Induction time also appears to be related to potency, with the earliest median time being 472 days with MCH and 594 days with the process residue.

TABLE 6

Squamous Cell Carcinoma Incidence and Induction Times with Carcinogens and Chromium Compounds

Material*	Number of animals surviving for 150 days†	Carcinoma incidence (%)	Median cancer time (days)	Range of time (days)
Methylcholanthrene	91	29.7	472	228–732
Benzpyrene	94	17.0	525	200–981
Calcium chromate	94	6.4	550	386–671
Process residue	93	1.1	594	

*50-50 mixture of material and cholesterol.

†One percent probability of recognizing a cancer earlier than this time (P = 0.01).

Implantation and Intubation Studies with Hamsters

Lung cancer studies have been extended to the hamster to provide another species which may demonstrate differences in sensitivity or production of other types of cancers. Table 7 shows the lung cancer incidence obtained with intrabronchial pellet implants in hamsters. Forty-seven carcinomas were observed in the 99 animals exposed to MCH, and 63 in the 97 animals exposed to BP. These findings include squamous cell carcinomas, adenocarcinomas, mixed (squamous and adenocarcinomas), and undifferentiated carcinomas. With both

TABLE 7

Intrabronchial Pellet Implants in Hamsters: Carcinogen Series with Methylcholanthrene and Benzpyrene

Type of implant	Number of animals	Number of lung cancers
Wire mesh controls	46	0
Methylcholanthrene	99	47
Benzpyrene	97	63

MCH and BP, squamous cell carcinomas occurred in highest incidences (18 and 28%, respectively) and showed the shortest mean induction times. Mixed carcinomas showed the lowest incidences (5 and 5%, respectively) and the longest mean induction times.

Our experiences with the endotracheal intubation technique in the hamster involved the use of repeated weekly doses of aqueous colloidal suspensions. This differed from Saffiotti's approach[2,3] in that only pure materials were used. Animals receiving BP intubations showed no change of significance other than bronchioalveolar metaplasia. In contrast, however, MCH resulted in a significant carcinoma incidence. With MCH, 82% of the animals surviving beyond 16 weeks developed lung cancer. The majority of the cancers were poorly differentiated adenocarcinomas, many having multifocal origin. The pathologic findings and the histologic progression of the changes are of special interest because of their similarity to human cancer.

At higher dose levels, there has been an increased incidence of early squamous cell carcinomas. In a group of 37 hamsters receiving 13 biweekly instillations of 5 mg MCH, 5 of the 11 cancers observed were squamous cell carcinomas. These appeared between 75 and 147 days. This early appearance is of importance in view of our experience with pellet techniques in the rat and hamster.

The studies that I have discussed so far have demonstrated the significance of the role of dosage in relation to the induction of cancer. The successful procedures are associated with local tissue injury as well as higher local dosage to the bronchial mucosa. Both mechanical injury and chemical irritation have been shown to enhance cancer incidence from chemical carcinogens. Pre-existing disease may lead to an increased likelihood of lung cancer in man. Additional factors potentiating the action of chemical carcinogenesis could operate by depressing clearance mechanisms in the lung, thus creating increased local carcinogen concentration, by providing groups of rapidly regenerating cells, by enhancing sensitivity following injury, or by more subtle intracellular mechanisms. Studies at this laboratory have demonstrated the rapid clearance of carcinogens following intratracheal instillations and inhalation exposures.[1] More

recently, Katz *et al.*[24] have demonstrated that the disappearance of BP from the lungs of hamsters is more rapid than the clearance of MCH. Kuschner[3] has suggested that the trauma induced by the pellet or operative techniques

> may well produce alterations in the bronchial mucosa which render it more susceptible to the action of carcinogens. There may well be a reason to believe that the florid proliferation and regeneration that follows trauma furnishes a more suitable cellular substrate for carcinogenic activity. Further, an area of traumatically altered or nonspecifically damaged bronchial epithelium may interfere with normal bronchial clearing mechanisms with resulting stasis and a selective increase in local dosage.

INHALATION TECHNIQUES

Our current inhalation experiments relate to the effects of irritants, carcinogens, and combinations of the two. Paralleling the development of these studies, we have also engaged in a program of developing a coordinated inhalation system at our Sterling Forest laboratories. I would like to spend a few minutes presenting the highlights of this system before proceeding with the studies.

Coordinated Inhalation System

Key features of this inhalation system are a variety of new developments in exposure chamber design, control equipment, and a centralized exhaust and air-cleansing system. The inhalation system provides capacity for more than 20 exposure units constructed of stainless steel, glass, and/or plastic. Each unit is functionally independent, with sufficient flexibility in feed and control accessories to provide for a variety of gas and/or aerosol atmospheres. Additional provisions permit each unit to be modified for shielding, internal decontamination, and maximum air cleansing with absolute filters and charcoal beds.

The exposure units range from small test systems (10- to 20-liter capacity) for range-finding studies to large units (1.3 cubic meters) for acute and chronic inhalation studies. Flexibility of internal construction of the larger units permits exposure of a variety of species, including dogs and monkeys.

All units are operated as dynamic systems, with provisions made for maintaining slight negative pressures relative to the atmosphere (0.1–1.0 inches of water). The airflow from each chamber is measured by an orifice meter connected to a differential Magnehelic pressure gauge. Control of airflow and static pressure are maintained by means of manually operated gate valves. Intake air is temperature conditioned and precleansed by passage through absolute filters. Airflow can be varied from 2–10 liters per minute for small units and from 2–20 cubic feet per minute for larger units. The rate of airflow and the temperature of entering air can be balanced with the requirements of the contaminant feed rate and the heat generated by the confined population of animals exposed. Total animal volumes are maintained at less than 5% of

chamber volumes to meet thermal balance requirements. Since the amount of air required for heat removal is usually far in excess of the volume needed for oxygen-carbon dioxide balance, this constitutes the practical lower design limit for airflow. A 5% loading of animals efficiently fills the entire main body of the chamber cages. Additionally, uniformity of exposure in terms of air distributions and concentrations can be well maintained at this level of loading.

The design of the equipment developed is based on the experience of this laboratory and previous experiences at the University of Rochester.[25] The exposure units are fundamentally derived from the hexagonal prism described by Wilson and Laskin.[26] This design provides uniform distribution of concentrations and particle sizes, as well as ease of maintenance. The stainless steel and plastic construction provides complete visualization of animal response to exposure at all times. The mixture of air contaminants is introduced into a short "T" section at the top of each chamber; this provides for uniformity of mixing with diluent intake air. Exhaust air is drawn centrally from the bottom of each chamber and passes through several stages of cleansing before entering the exhaust discharge system.

Figure 15 illustrates two of the large inhalation chambers used for exposure of animals to carefully controlled atmospheres of particulates and/or gases. These units stand approximately 9 feet high, and have a total volume of 1.3 cubic meters and an effective animal exposure volume of 1.0 cubic meters. Experience with this unit has shown that effective exposure can be given to a population consisting of 75 rats, 35 guinea pigs, and 4 dogs, simultaneously. Figure 16 shows a smaller version of this type inhalation chamber. The chamber and its controls are approximately 4 feet high, and it has a total volume of 112 liters and an effective animal exposure volume of 86 liters.

For the carcinogen and combined carcinogen-irritant exposure studies, an isolation-exposure system, shown in Fig. 17, was developed. Also illustrated in the figure are sampling equipment and a continuous gas analyzer. This system consists of three dry boxes isolated by internal sliding doors. Two large boxes are animal living quarters with facilities for lifetime isolation of control and treated animals. Food and animal wastes are transferred to the dry boxes by means of a pass box. A third large dry box encloses a small inhalation exposure unit. Mounted above this box is a dry box and pass box for the handling of the contaminant feed. Mounted below is an additional dry box for exhaust chamber cleansing. All controls are external, including dry-box airflows, chamber airflow, and air conditioning and feed pressures. Since this system is designed for highly hazardous materials, a number of special air cleansing features have been incorporated: these include a water scrubber, electrostatic chamber-air cleansing, and absolute filters. The enclosed animal colony is maintained under conditions of slightly negative pressure (0.5 inches of water). An air volume of 25 cubic feet per minute maintains temperature balance and is controlled to a temperature of 76°F. All intake air and water is prefiltered.

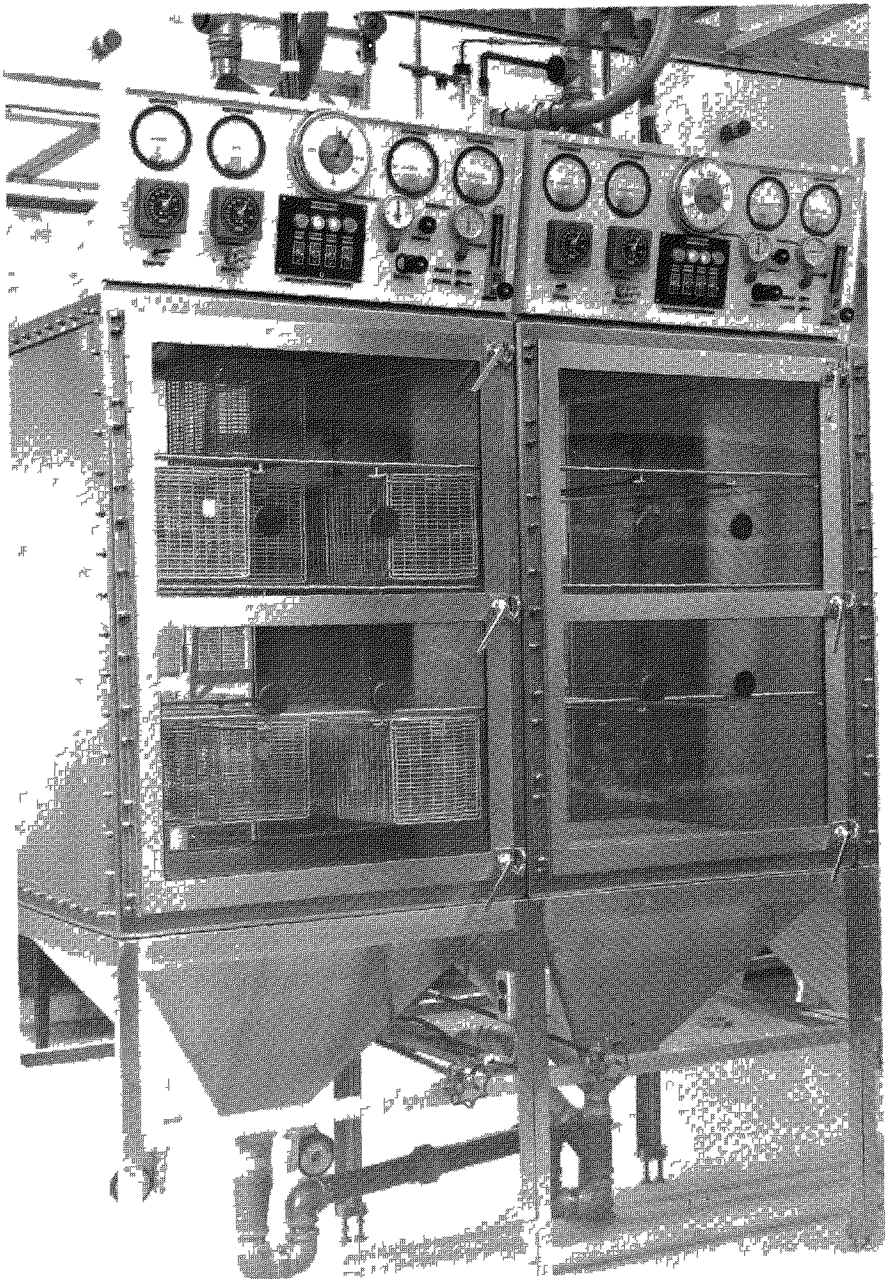

Fig 15 – Large inhalation exposure chambers These units provide for the exposure of animals to carefully controlled atmospheres of particulates and/or gases

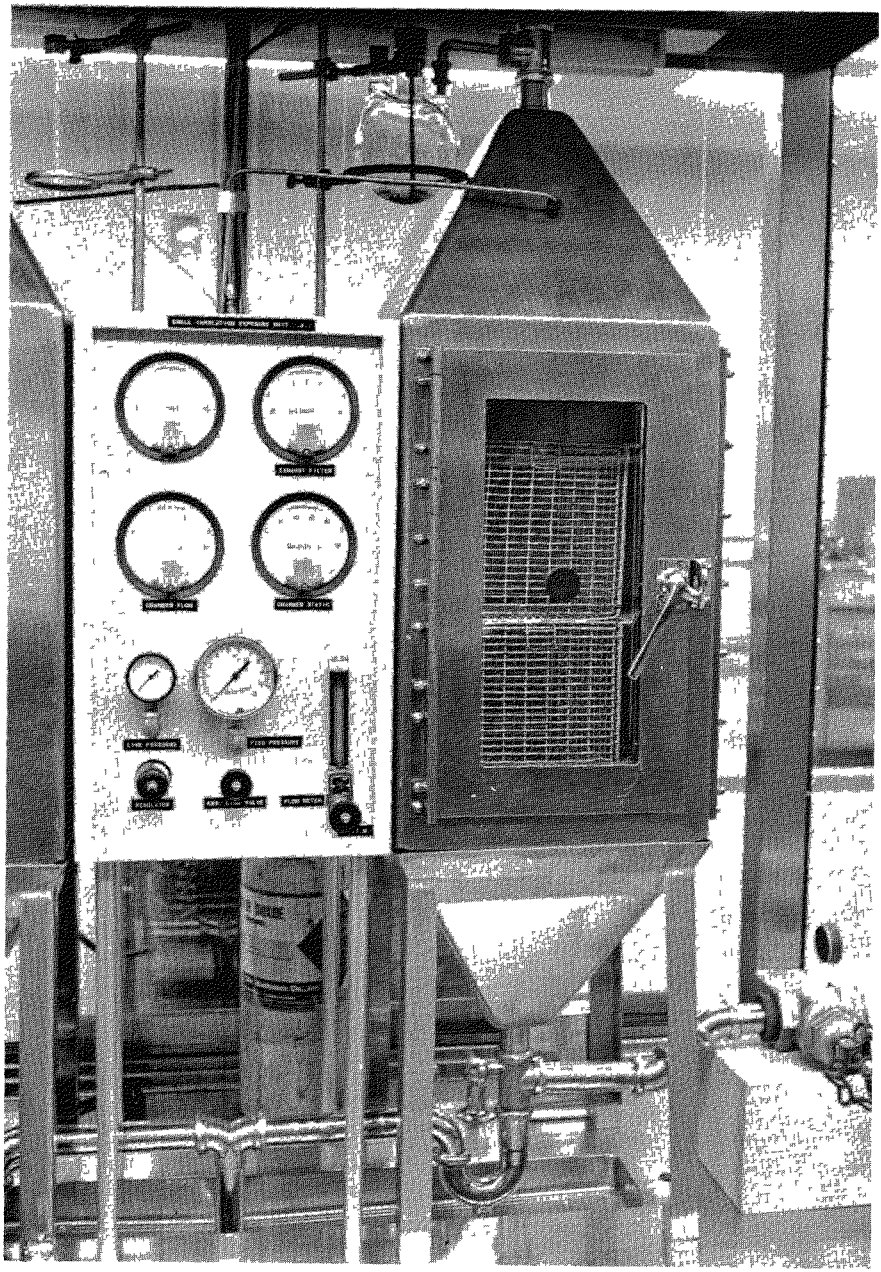

Fig. 16 – Small inhalation chamber for the exposure of animals to contaminated atmospheres.

Fig. 17 – Isolation-inhalation exposure system providing for remote and dry box handling of animals and inhalation equipment.

Inhalation Exposures to Sulphur Dioxide and Benzo[*a*] pyrene

For our initial studies with respiratory irritants we selected sulfur dioxide because of its implications in community air pollution problems. Inhalation exposures of rats to sulfur dioxide atmospheres were performed on a schedule of 6 hr per day, 5 days per week, for periods of up to 16 weeks. The pattern of exposures, the concentrations, and the cumulative mortality observed are given in Table 8. All exposure-induced mortality was observed within 3 weeks. Up to the 12th day, incidences ranged from 4.7% at 10 ppm to 87.2% at 567 ppm.

Gross damage and clinical symptoms were most marked at 567 ppm and absent at 10 ppm. Lungs of animals dying during exposure showed significant congestion, edema, and pneumonia. Tracheitis, bronchitis, ulceration, and marked mucosal proliferative activity of the trachea and bronchi were of particular interest. These regenerative changes included loss of cilia, hyperplasia, and stratification. The frequency of such pathological changes at the 105 ppm level are given in Table 9.

TABLE 8

*Exposure Levels and Cumulative Mortality of Rats Exposed to Sulfur Dioxide Atmospheres**

Mean concentration (ppm)	Group	No. of animals	Exposure period (days)	No. of exposures	Percent cumulative mortality at various days after start of exposure				
					8	12	22	56	113
567	Exptl.	47	12	7	44	87†			
	Control	20			10	10†			
105	Exptl.	45	22	15	10	15	40†		
	Control	20			5	5	5†		
51	Exptl.	48	112	77	12	12	18	18	18
	Control	20			5	10	10	10	10
10	Exptl.	48	113	78	2	5	5	5	5
	Control	20			0	0	10	15	15

*Exposure schedule – 6 hr/day, 5 days/week.
†Experiment terminated.

TABLE 9

*Pathological Changes in Rats Dying During Exposure to Sulfur Dioxide Atmospheres at 105 ppm**

Pathological changes	Percent of animals showing changes
Trachea	
Tracheitis	100
Purulent tracheitis	9
Ulceration	9
Regenerative change	72
Bronchi	
Bronchitis	72
Purulent bronchitis	45
Ulceration	9
Regenerative change	54
Lungs	
Congestion	57
Edema	43
Pneumonia	57

*Only one control animal died during the exposure period.

Serial sacrifice studies made during and at termination of the exposure periods reflect the gross action of sulfur dioxide as an acute respiratory irritant. Tracheitis was found in virtually all animals at all levels, and high incidences of bronchitis, congestion, and pneumonia were seen at 105 ppm and 567 ppm. Significant ulceration was observed in the trachea and bronchi at 567 ppm. Regenerative changes occurred at all exposure levels in the trachea, with incidences reflecting severity of exposure. Regenerative changes in the bronchi were most marked at the 105 and 567 ppm levels. Figure 18 illustrates regenerative hyperplasia and early metaplasia seen 4 days after exposure to 105 ppm of sulfur dioxide. Changes observed in controls reflected the degree of infection endemic in laboratory rats. Two weeks after cessation of exposure, the mucosa, for the most part, reverts to normal.

Combined carcinogen and irritant inhalation studies were carried out in the isolation-exposure system. One of the animal housing units was modified to permit gas exposure. In this unit, 24 rats and 20 hamsters were exposed to 10 ppm sulfur dioxide for 6 hr per day, 5 days per week. The second housing unit was used as a control. It contained 24 rats and 20 hamster living in a prefiltered fresh air atmosphere. Four groups of animals from each of the living units were transferred to the internal inhalation chamber for combined carcinogen-irritant exposures (10 mg/m^3 benzo[*a*]pyrene aerosol-3.5 ppm sulfur dioxide). Each

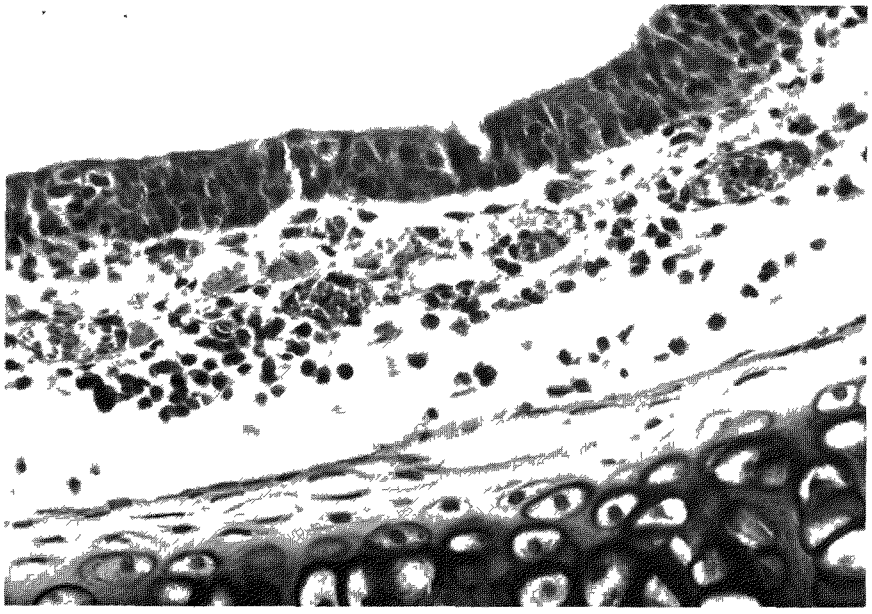

Fig. 18 – The effect of SO_2 on tracheal and bronchial mucosa of rats. Regenerative hyperplasia and early metaplasia are shown 4 days after daily 1 hr inhalation of 100 ppm of SO_2. (265X; reduced 28%)

group was exposed to the carcinogen for 1 hr per day, 5 days per week In this experiment, which spanned a period of 794 calendar days, animals received up to 494 exposures to the carcinogen-irritant atmospheres and up to 534 exposures to irritant atmospheres The types, pattern, and periods of exposure to the irritant and carcinogen-irritant atmospheres are given in Table 10

TABLE 10

*Inhalation Exposures of Rats and Hamsters to Sulfur Dioxide and/or Benzo[a]pyrene (BP) Atmospheres**

Exposure type	Exposure pattern, 5 days per week		Exposure period, 794 calendar days	
	Irritant, 6 hr/day	Carcinogen-irritant, 1 hr/day	Exposure (days)	
			I	CI
A (fresh air)				
A + CI		10 mg/m^3 BP + 3 5 ppm SO_2		494
I	10 ppm SO_2		534	
I + CI	10 ppm SO_2	10 mg/m^3 BP + 3 5 ppm SO_2	534	494

*Isolated living quarters contained prefiltered, conditioned fresh air during nonexperimental periods

TABLE 11

Inhalation Exposures to Sulfur Dioxide and/or Benzo[a]pyrene Atmospheres

Exposure type	Number of animals	Cumulative mortality of rats				
		Weeks of exposure				
		32	48	64	80	98
A	3	0	0	0	1	2
A + CI	21	0	0	1	4	12
I	3	0	1	1	2	2
I + CI	21	0	0	0	3	8

The survival of rats in these studies was exceptionally good, with significant mortality occurring only after 80 weeks (Table 11) Hamster mortality reflected toxic reactions to the exposures and was complete by 98 weeks Although no significant pathology was found in the hamsters, the rats showed findings of squamous cell carcinomas (Table 12) Of the 21 rats that lived in fresh air and were exposed to the carcinogen-irritant atmosphere, two developed squamous cell carcinoma of the lung by 655 and 698 days Both of these animals also showed renal metastasis Figures 19a and b are gross photographs of the lung

TABLE 12

Inhalation Exposures to Sulfur Dioxide and/or Benzo[a]pyrene Atmospheres

	Significant pathological findings in rat lungs		
Exposure type	Number of animals	Advanced squamous metaplasia*	Squamous cell carcinoma*
A	3	0/3	0/3
A + CI	21	1/21	2/21
I	3	0/3	0/3
I + CI	21	2/21	5/21†

*Expressed as a ratio of tumors found to animals observed.
†Secondary squamous cell carcinoma in kidney.

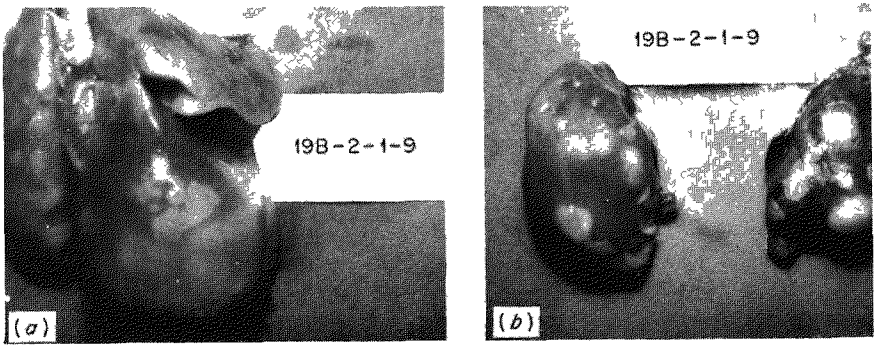

Fig. 19 – Gross photographs of (a) squamous cell carcinoma in the lung of a rat exposed to a combination of SO_2 and benzo[*a*]pyrene by inhalation; (b) metastasis of the tumor to the kidneys. (From Kuschner[3])

carcinoma and metastasis to the kidneys in one of these animals. In this group, a third animal showed advanced squamous mataplasia at 623 days.

Of the 21 animals exposed to both sulfur dioxide and carcinogen-irritant atmospheres, five were found with squamous cell carcinoma of the lung at 547, 603, 625, 655 and 794 days. Figure 20 illustrates a keratinizing squamous cell carcinoma found in this study. The earliest cancer showed local extension (Fig. 21) and metastasis to the kidney (Fig. 22). Two additional animals showed advanced squamous metaplasia at 485 and 715 days. These findings are of special significance since both sulphur dioxide and benzo[*a*]pyrene have been demonstrated to be present in the atmospheres of major cities, and thus may have implications in our current air pollution problems.

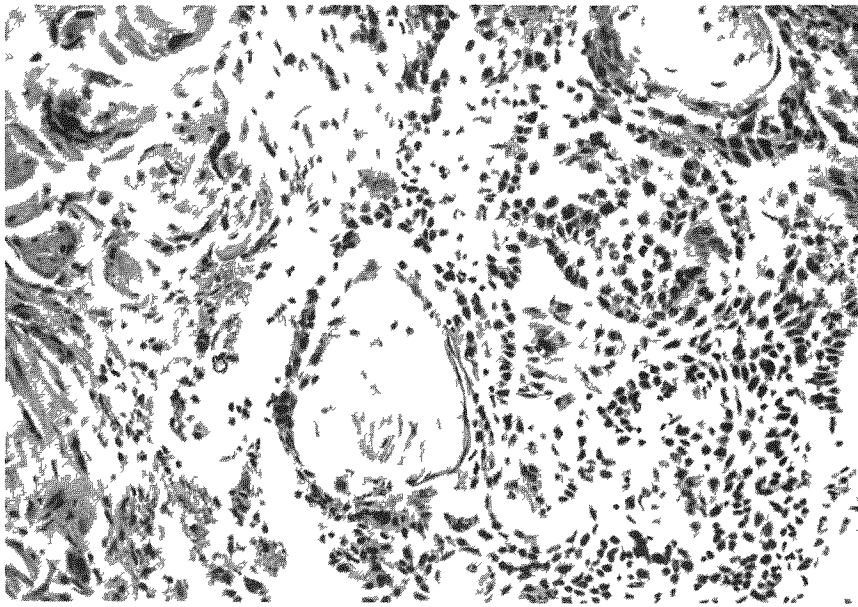

Fig 20 Photomicrograph of a keratinizing squamous cell carcinoma of the lung induced in a rat by inhalation of benzo[*a*]pyrene and SO_2 (265X reduced 28%)

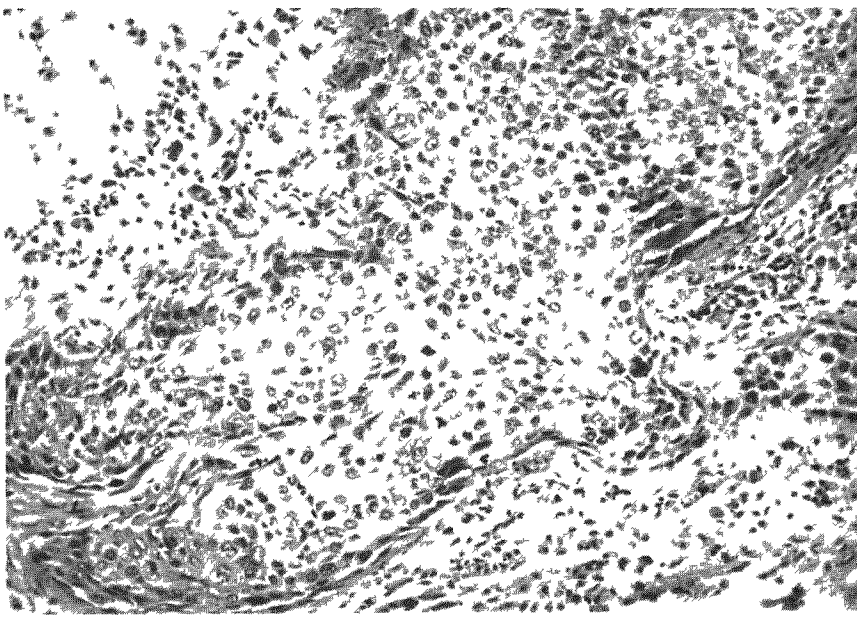

Fig 21 Invasion and permeation of pulmonary vein by squamous cell carcinoma (265X reduced 28%)

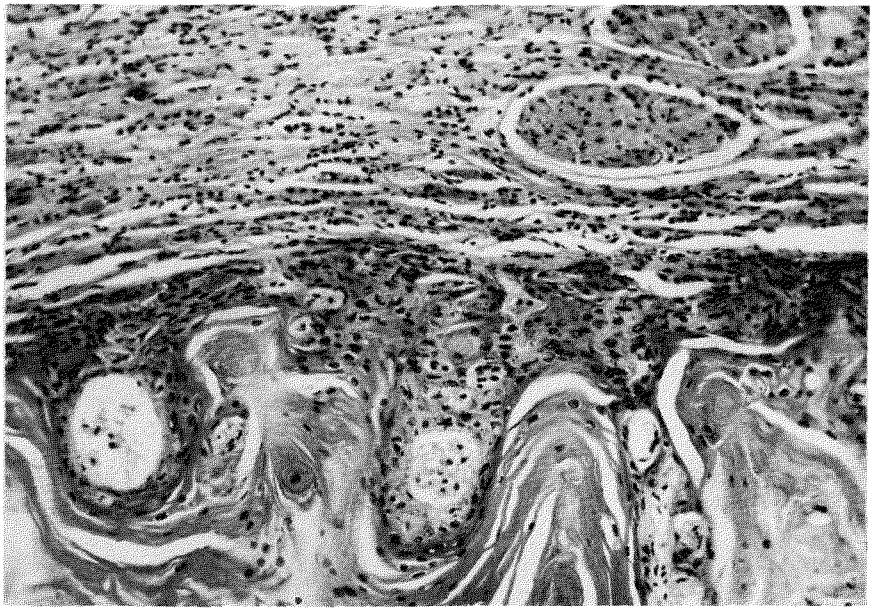

Fig. 22 – Renal metastasis in a squamous cell carcinoma of rat lung induced by inhalation of benzo[*a*]pyrene and SO_2. (265X; reduced 28%)

To summarize, I would like to quote Dr. Kuschner's recent comments[3] on our studies:

> In conclusion, then, we believe we have at our disposal now a series of animal models of lung cancer which quite closely simulate lung cancer in man. These have not been available heretofore. Bronchial mucosal changes and tumors of bronchogenic origin comparable to human lung cancer can be induced in a number of rodent species. Indeed, one gets the feeling that rats are more like man than some men. These model systems have begun to permit us to identify the role in pulmonary carcinogenesis of the many and varied substances to which man is exposed. The experimental approach has contributed insights into the importance of combined effects. Finally, a study of the experimental models has illuminated the morphogenesis of the malignant transformation and helped make understandable the variety of mucosal alterations we observe in man.

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REFERENCES

1 M Kuschner, S Laskin, E Christofano, and N Nelson, Experimental carcinoma of the lung In, *Proceedings of the Third National Cancer Conference,* p 485 J B Lippincott Company, Philadelphia, 1957
2 H B Andevont, Pulmonary tumors in mice IV Lung tumors induced by subcutaneous injection of 1,2,5,6-dibenzanthracene in different media and by its direct contact with lung tissue *Pub Health Rep* 52 (1937) 1584
3 M Kuschner, Causes of lung cancer, J Burns Amberson Lecture *Am. Rev Respirat Diseases* 98 4 (1968) 573
4 D J Finney, *Statistical Method in Biological Assay* Hafner Publishing Company, 1952
5 L C Miller, Biological assays involving quantal responses In, *The Place of Statistical Methods in Biological and Chemical Experimentation* Vol 52-6, p 903, E J DeBier *et al* (eds) Annals of the New York Academy of Sciences, 1950
6 H Lisco and M P Finkel, Observations on lung pathology following the inhalation of radioactive cerium *Fed Proc* , 8 (1949) 360
7 H Lisco, Autoradiographic and histopathologic studies in radiation carcinogenesis of the lung *Lab Invest* 8 (1959) 162
8 J K Scott and R Thomas, Polonium induction of pulmonary tumors following intratracheal injection *University of Rochester Atomic Energy Project Quarterly Review,* July–Sept (1957) p 30
9 H Cember and J A Watson, Bronchogenic carcinoma from radioactive barium sulfate, *A M A Arch Ind Health,* 17 (1958) 230
10 L A Temple, D H Willard, S Marks, and W J Bair, Induction of malignant tumors by radioactive particles *Nature* 183 (1958) 408
11 H Cember, J A Watson, and A A Spritzer, Bronchogenic carcinoma from radioactive cerium *A M A Arch Ind Health,* 19 (1959) 14
12 H Cember and J A Watson, Carcinogenic effects of strontium 90 beads implanted in the lungs of rats *Am Ind Hyg Assoc J* 19 (1958) 36
13 O Gates and S Warren, The production of bronchial carcinomas in mice, *Am J Pathol* 36 (1960) 653
14 B Altshuler Dosimetry from a ^{106}Ru coated platinum pellet *Radiation Res* 9 (1958) 626
15 S Laskin M Kuschner, N Nelson, B Altshuler, J Harley, and M Daniels Carcinoma of the lung in rats exposed to the beta radiation of intrabronchial ruthenium^{106} pellets 1 Dose-response relationship *J Natl Cancer Inst* 31 (1963) 219
16 Committee on the Pathologic Effects of Atomic Radiation, Effects on Inhaled Radioactive Particles Publication No 848, National Academy of Science – National Research Council, 1961
17 J K Wagoner, J E Archer, F E Lundin, D H Holaday, and J W Lloyd, Radiation as the cause of lung cancer among uranium miners *New Engl J Med* 273 (1965) 181
18 S Laskin, M Kuschner, B Altshuler, and N Nelson, Tissue reactions and dose relationships in rats following intrapulmonary beta radiation, *Health Phys* 10 (1964) 1229
19 W M Gafafer *et al* Health of workers in chromate-producing industry Health Service Publication No 192, U S Public Health Service, 1953

20 W Alwens, Lungenkrebs durch arbeit in chromat herstellenden betrieben In, *Bericht uber den VIII Internat Kongress for Unterfallmedizin v Berofskrankheiten* Vol 2, p 973 Leipzeig, Thieme, 1939
21 W W Payne, Production of cancers in mice and rats by chromium compounds *A M A Arch Ind Health* 21 (1960) 530
22 W C Heuper and W W Payne, Experimental studies in metal carcinogenesis *Arch Environ Health,* 5 (1962) 445
23 U Saffiotti, F Cefis, and L H Kolb, A method for the experimental induction of bronchogenic carcinoma *Cancer Res* 28 (1968) 104
24 G V Katz, S Laskin and M Kuschner, Pulmonary clearance of carcinogens in hamsters, A I H A Ind Health Conf Denver, Colorado, 1969 *Am Ind Hyg Assoc J*, 30 2 (1969) 109 (Abstract)
25 H E Stokinger *et al*, Toxicity following inhalation In, *The Pharmacology and Toxicology of Uranium Compounds,* National Nuclear Energy Series, Division VI, Vol 1, p 694, C Voegtlin and H C Hodge (eds.). McGraw-Hill, Inc , New York 1949
26 R H Wilson and S Laskin, Improved design of an animal inhalation exposure unit In, AEC Project Report UR-116, p 80, 1950

DISCUSSION

R. G. Thomas. Your curves of incidence versus microcuries [Fig 7 in text] stop at about 65%, and I assume that's because if you go higher in microcuries or radiation dose, incidence decreases because the animals die before you see anything

S. Laskin: That's what happened in the first series of experiments

Thomas: My question concerns the abscissas on your probit plots Now are they, the way you use them, associated with the point of inflection that you would get by giving a higher dose, so that your incidence would decrease? Where do the abscissas you have on your probit plots come from?

Laskin: The plots were incidence in percent on the probit plot (accumulated percentage). It's exactly the same trick that you do in an LD_{50} study In other words, if you give a very high dose, you kill 100% of the animals Incidence of cancer versus dose, that's all

But back to your first point Although an incidence of 65% appeared in these data of the initial study, later serial sacrifices suggested that the incidence for radioactive pellets would essentially approach 100% It is a question of backtracking and redoing the experiment, and we are fairly convinced that with, say, implants in the order of 1 to 5 μCi we can easily duplicate this, and we have done so in several instances

F. J. C. Roe: I should like to offer a bouquet to Professor Laskin and the whole team at NYU for work which I regard as pioneer studies in the field I should like to ask Dr Kuschner some questions

I've always rather doubted the need to produce squamous carcinomas as an essential part of the model for human lung cancer Were kerato-hyaline granules present in the squamous carcinomas produced experimentally in your rats

and/or in the squamous metaplasia? I believe that in the lesions produced in mice by Kotin and Falk with ozonized gasoline and influenza virus, these granules were not present Is this true? And if so, is it important? Does it in any way invalidate this model for human lung cancer?

M. Kuschner The question raised by Dr Roe is an important one and might well have been raised earlier

I feel very strongly that the term *tumors of the lung* is an unfortunate one Even *cancer of the lung* is an unfortunate term I think all of us here know that cancer is a disease of a tissue, not of an organ There are particular determinants that relate to the production of a bronchogenic carcinoma that probably do not hold for tumors of more peripheral sites

I believe that we can study the mechanism of malignant transformation and the factors that relate to it in any part of the body and in any tissue Perhaps peripheral lung tumors are a convenient method of doing this, but they don't relate to the immediate problem, that is, what are the particular factors that go into the induction of and the pathogenesis of – bronchogenic carcinoma in humans

It is for this reason that I think the induction of pulmonary adenomas, or adenocarcinomas, is not pertinent to the lung tumor problem They are pertinent to the tumor problem, but not to specific induction of the lung tumor that we are concerned with, bronchogenic carcinoma

I think it might be important, too, in regard to Dr Sanders' presentation [these *Proceedings,* pp 285–303], to point out that as far as I know the tumors produced by J F Park and by C L Yuile were all peripheral tumors of alveolar origin Some of the dosage inconsistencies between alpha and beta emitters which did produce bronchogenic carcinoma might perhaps be explained by the fact that we are dealing with a different tissue

In regard to the kerato-hyaline granules, I think my own experience has been that they are not characteristic of squamous tumors or of squamous change in mucous membranes One loses kerato-hyaline granules, even in the epidermis, when tumor transformation takes place

THE ROLE OF TOPICAL AND SYSTEMIC FACTORS IN EXPERIMENTAL RESPIRATORY CARCINOGENESIS

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ABSTRACT

During the past decade considerable effort has been devoted in the authors' laboratories to the development of experimental models for the study of the pathogenesis of lung cancer in experimental animals. The Syrian golden hamster has been the species most extensively used. It proved markedly susceptible to various respiratory carcinogens and conveniently refractory to chronic pulmonary infections. Direct administrations of polynuclear hydrocarbons into the respiratory tract were first performed with colloidal gelatin suspensions; most subsequent work made use of finely dispersed ferric oxide in saline as a carrier for crystalline carcinogen particles. High incidences of bronchogenic carcinomas were obtained by the latter method, using benzo[*a*]pyrene and ferric oxide. The tumors most frequently observed were squamous cell carcinomas. Repeated intratracheal instillations were most effective, although some tumors could be induced even by a single administration. Direct dose-response correlations were demonstrated by varying the number of administrations, their frequency, and the levels of each dose given for different lengths of time. The systemic carcinogen diethylnitrosamine was used for dose-response studies after subcutaneous administrations to adult or newborn hamsters. The tumor response was consistently localized in the upper respiratory tract; only a few tumors were seen in the bronchi and lungs. When such systemic exposure was combined with the intratracheal administration of benzo[*a*]pyrene and ferric oxide or even of ferric oxide alone, a marked synergistic tumor response was observed in the lower respiratory tract. The role of the time sequence, specificity, and dose of the factors involved in this synergistic action is presently under study. Other topical and systemic respiratory carcinogens have been investigated in hamsters, mice, and rats.

In recent years a considerable effort has been devoted to the development of experimental models by which respiratory tumors, particularly bronchogenic carcinoma, could be included in experimental animals. At the present time a few such experimental models have been successfully and reproducibly developed,

thus permitting us to study the causative agents, conditioning factors, and pathogenetic mechanisms of this type of cancer.

The field of experimental respiratory carcinogenesis was reviewed and discussed at the International Conference on Lung Tumours in Animals, held in June 1965 at the University of Perugia.[1] The main experimental models for pulmonary carcinogenesis have been recently reviewed.[2,3]

A series of studies on experimental respiratory carcinogenesis has been conducted in our laboratories over a period of about 15 years. Most of this work made use of the Syrian golden hamster as the experimental animal. This species was studied for its general responsiveness to chemical carcinogens.[4] Polynuclear hydrocarbons, most frequently benzo[*a*]pyrene (BP), were first used for topical administration into the respiratory tract by intratracheal instillations. Various particulate materials, mainly metal oxides, were studied using the same mode of administration, alone or as carriers of polynuclear hydrocarbon carcinogens. The systemic respiratory carcinogen diethylnitrosamine (DEN) was studied for its dose-response effects in adult and newborn hamsters by subcutaneous administration. Other *N*-nitroso compounds, administered systemically to hamsters and rats, were also found to induce respiratory tumors.[5,6] In our work of the last few years, considerable effort has been given to the study of the combined effects of topical and systemic factors in respiratory carcinogenesis.

A variety of morphological responses, including different tumor types ranging in site of origin from the nasal cavities through the tracheobronchial tract of the peripheral alveolar region, have been observed. The correlation between the specificity of the morphological tumor response and the mechanism of induction has received particular attention.

POLYNUCLEAR HYDROCARBONS BY INTRATRACHEAL INSTILLATION

Early studies by Della Porta, Kolb, and Shubik[7] showed that repeated intratracheal instillations of the potent carcinogen 7,12-dimethylbenz[*a*]-anthracene in a colloidal gelatin suspension could induce tumors of the larynx, trachea, and bronchi in Syrian golden hamsters. This and all subsequent work in our laboratory[2,3,8-19] as well as in others[20-33] has amply demonstrated that the hamster is indeed a species of choice for studies on experimental lung cancer, because of its lack of spontaneous lung tumors,[4,7,12,16,34] its marked resistance to pulmonary infections, and its responsiveness to different respiratory carcinogens.

An experimental method was subsequently developed for the administration of carcinogens by the respiratory route in hamsters using particulate carriers. This model reproduces many of the conditions present in the human exposure to inhaled carcinogens and has proved effective in inducing a high incidence of tumors of the respiratory tract.[11,12,14,18] In most of the experiments, BP was

dispersed in fine crystalline particles smaller than 1 μ and attached by surface adhesion to similarly fine particles of ferric oxide to produce a homogeneously mixed dust. The dust, suspended in saline, was administered by intratracheal instillation to hamsters. The procedures used for the preparation of the suspensions were previously described in detail.[2,11,12] A number of studies were carried out with this method, particularly on dose-response relationships in respiratory carcinogenesis.

Single doses were first tested in long-term bioassays using a single intratracheal instillation of 5 mg of BP mixed with 45 mg of ferric oxide in one group, and 37.5 mg of BP with 12.5 mg of ferric oxide in another group. The latter was the maximum single dose of BP that could be practically administered to hamsters. The incidences of respiratory tract tumors were 4% in the lower BP dose level group and 15% in the higher;[18] these results show that bronchogenic carcinomas and other respiratory tract tumors could be induced even by a single administration of BP and ferric oxide.

Different numbers of administrations were then tested. Two series of experiments were run separately using the same three dose levels for each. Two groups of hamsters thus received each of the following dose schedules: 5, 10, or 15 administrations of a weekly dose of 3 mg of BP and 3 mg of ferric oxide.[18] Decreasing numbers of administrations induced decreasing tumor incidences and increasing delays in the time of death of the animals with respiratory tumors.

Different dosage per administration was studied in four groups of hamsters, treated weekly for 30 weeks with 2.0, 1.0, 0.5, and 0.25 mg of BP mixed with an equal amount of ferric oxide.[16] A positive correlation was demonstrated between the tumor yield and the dose level of the individual administrations. Decreasing the dose level of each administration, with a consequent decrease in total dose, also resulted in a corresponding delay of tumor appearance.

Fractionated small doses were compared with single doses. As expected, lower fractionated levels of BP were found to be more effective than single larger doses. In a group receiving 0.25 mg of BP for 30 weeks the first bronchial tumor was detected in an animal that died at 22 weeks, after exposure to a total dose of 5 mg of BP; while in a group treated with 5 mg of BP as a single administration, we observed the first tumor at 52 weeks.[16,18]

Frequency of administration is another variable that was found related to tumor incidence. In two groups of hamsters receiving 2 mg of BP every second week for 30 weeks, final respiratory tumor incidences reached 20 and 23%, respectively.[9,35] Such incidences appear markedly low when compared with an incidence of 60% obtained in a group treated with the same total dose (30 mg) administered at *weekly* intervals 1 mg at a time for 30 weeks. The weekly frequency of administration proved more effective even with a total dose of only 15 mg of BP (administered weekly in 0.5-mg doses), giving a tumor incidence of 30%.[16]

On the other hand, when only five administrations were given, each of 3 mg of BP, spacing their frequency at intervals of 7 days or of 25 days made no

TABLE 1

*Respiratory Tract Tumors and Lesions in Hamsters Following Intratracheal Instillation of Benzo[a] pyrene and Ferric Oxide**

Number of animals autopsied, 887, number of tumor bearing animals, 253, total number of tumors, 359

Tumor sites	No of tumors†	Morphological types of tumors and lesions	No of tumors or lesions	Ratios of: Malignant tumors / Benign tumors	Squamous metaplasia / Squamous tumors	Squamous tumors / Total tumors	Squamous tumors / Adenomatous tumors	Squamous tumors / Anaplastic tumors
Larynx	14 (4%)	Squamous metaplasia, alone	4	6 0	0.4	0 8	5 5	
		Polyp	2					
		Carcinoma — Squamous cell	11					
		Carcinoma — Adenocarcinoma	1					
Trachea	115 (32%)	Squamous metaplasia, alone	46	0 9	0 6	0 8	2 9	16 0
		Papilloma, squamous cell	31					
		Polyp	29					
		Carcinoma — Squamous cell	49					
		Carcinoma — Anaplastic	5					
		Carcinoma — Adenocarcinoma	1					
Bronchi	181 (50%)	Squamous metaplasia, alone	20	9 0	0 2	0 8	1 7	2 1
		Papilloma, squamous cell	6					
		Polyp	1					
		Carcinoma — Squamous cell	82					
		Carcinoma — Anaplastic	43					
		Carcinoma — Adenocarcinoma	38					
		Adenoma	11					
Bronchioles and alveoli	49 (14%)	Squamous metaplasia, alone	55	0.75	5.0	0 3	0 26	3 0
		Adenomatoid lesion	156					
		Carcinoma — Squamous cell	9					
		Carcinoma — Anaplastic	3					
		Carcinoma — Adenocarcinoma	9					
		Adenoma	26					
		Adeno-squamous tumor	2					

*Cumulative analysis of results pooled from experiments in Syrian golden hamsters of both sexes treated with intratracheal instillations of benzo[*a*] pyrene and ferric oxide at different dose schedules.

†Number of tumors at each site and their percent out of total number of tumors

detectable difference in the response; however, the low-level response so obtained may have been inadequate to detect a relatively small difference.

Cumulative analysis of tumor types, based on the cumulative results of these experiments, is presented here in an attempt to evaluate the relative frequency of various types of respiratory tumors and their distribution in the different segments of the respiratory tract. The detailed morphology of the tumors and lesions observed has been previously described.[12,14,17,18]

The tumors can be classified by site of origin in tumors of the larynx, trachea, bronchi (stem bronchi and segmental bronchi) and of the peripheral or distal airways (bronchioles and alveoli). The morphological classification so far used in our studies includes: *squamous metaplasia* (patches of squamous differentiation, in some cases showing keratinization); *polyps* (benign papillary tumors showing a columnar mucous epithelium); *papillomas* (benign papillary tumors showing squamous epithelium); *carcinomas* (epithelial tumors showing anaplastic morphology and infiltration, with three types of differentiation: squamous-cell carcinomas in which the epidermoid differentiation is prevalent, adenocarcinomas in which the adenomatoid columnar mucous epithelium is prevalent, and anaplastic carcinomas in which the prevalent pattern is that of undifferentiated anaplastic cells, although small areas may be found in these tumors showing either a squamous or a mucous differentiation or, in some instances, both in the same tumor); *adenomas* (benign tumors with columnar mucous epithelium); and *adeno-squamous tumors* (benign tumors showing both types of differentiation).

Table 1 presents a cumulative analysis of tumor types induced by intratracheal instillations of BP and ferric oxide from pooled data that have been analyzed so far in our laboratory. The experimental details and results of individual experiments have been reported separately.[9,12,14,16–18,35]

Out of a total of 887 hamsters examined, 253 developed tumors of the respiratory tract; in many instances the tumors were multiple, resulting in a total number of 359 tumors. The tumors were localized mainly in the bronchi (50%) and in the trachea (32%); the larynx and the bronchiolo-alveolar region developed a much lower number of tumors – 4% and 14%, respectively. The ratio of tumors diagnosed as malignant to those considered benign was higher in the bronchi (9.0) than in the larynx (6.0), trachea (0.9), and bronchioles-alveoli (0.75). Concerning the types of tumors, the trachea and the bronchi showed the highest number of squamous tumors: papillomas and squamous cell carcinomas in the trachea and almost exclusively squamous cell carcinomas in the bronchi; in these two segments of the respiratory tract the ratios of squamous to adenomatous tumors (polyps, adenocarcinomas, and adenomas) were 2.9 for the trachea and 1.7 for the bronchi. The ratio in the larynx was also high (5.5), while in the bronchiolo-alveolar region it appeared low (0.26). The ratios of squamous to anaplastic tumors were 16 for the trachea, 2.1 for the bronchi, and 3 for the periphery of the lung.

These data indicate that the most common type of tumor was the squamous cell carcinoma of the bronchi; the bronchi showed relatively high incidences of anaplastic carcinomas and adenocarcinomas, some of which contained areas of squamous metaplasia; the adenomas appeared to originate from the mucosa of the small bronchi. The trachea also showed a high frequency of squamous tumors of which 61% were carcinomas. In addition, the trachea developed benign polyps and a few anaplastic carcinomas; the only adenocarcinoma observed could have arisen from the glands of the submucosa. Tumors of the larynx accounted only for 4% of the total number of respiratory tumors; 11 out of 14 were squamous cell carcinomas.

Most of the tumors developing in the periphery of the lung (bronchioles and alveoli) were of the adenomatous types, mostly histologically benign adenomas, and a few adenocarcinomas. In addition, a few squamous, anaplastic, and adeno-squamous tumors were observed.

Areas of squamous metaplasia without tumors appeared more often in the peripheral segments and in the trachea than in the bronchi, where squamous cell carcinomas were most often observed. This correlation could be explained by a more frequent development of squamous carcinomas from squamous metaplasia in the bronchi, as has been described in different experimental models.[12,36–39]

TABLE 2

*Respiratory Tract Lesions in Hamsters Following Intratracheal Instillations of Ferric Oxide**

Treatment		No. of animals autopsied	No. of animals with lesions of the respiratory tract		
Total dose (mg)	Dose schedule		Tumors	Squamous metaplasia	Bronchiolar adenomatoid lesions
50	Single dose	190	0	0	4
45	3 mg/week	41	0	0	0
60	2 mg/week	97	0	0	1

*Cumulative analysis of results pooled from experiments in Syrian golden hamsters of both sexes treated with intratracheal instillations of ferric oxide.

The bronchiolar adenomatoid lesions are still somewhat difficult to interpret. They were reported by Herrold and Dunham[28] in their experiments on hamsters treated with instillations of BP in Tween solution, by Shabad in rats,[40] and also by Clarke *et al.*[41] in dogs after exposure to $^{239}PuO_2$ aerosols. In a few cases we observed these lesions in hamsters after instillations of ferric oxide alone (Table 2);[9,12,14,18] however, their number is greatly increased by the administration of carcinogens. In some cases there are intermediate stages linking the peripheral proliferative lesions with clearly neoplastic areas.[12,14]

The higher susceptibility of the trachea and bronchi to tumor induction by respiratory administration of polynuclear hydrocarbons with particulate carriers is indicated by the earlier appearance of tumors in these regions than in the bronchiolo-alveolar region.[12,16,18] The morphology and topography of the induced lesions remarkably resemble the classical picture of their counterparts in man. In addition, each segment of the respiratory tract shows a tumor incidence directly related to the dose and period of administration.[16,18]

In the experimental model we have discussed so far, the role of ferric oxide, administered together with the carcinogen, is still not properly assessed. In over 300 hamsters treated intratracheally with ferric oxide alone, no respiratory tumors were found; the only sign of a proliferative response were a few peripheral adenomatoid lesions (Table 2).

An essential role of the ferric oxide particles is their capacity to facilitate the penetration and retention of the carcinogen in the pulmonary tissues;[2,11,12] this may be comparable to the role of other particulates – such as gelatin, casein, and India ink powder – used in other experiments.[7,39,42] However, a more complex function is suggested for the role of particles in the induction of respiratory tumors in the light of some of our more recent experiments that will be discussed below. Hamsters pretreated with relatively low doses of DEN responded to subsequent intratracheal instillations of ferric oxide with a much higher incidence of lung tumors than did the hamsters treated with DEN alone.[9] It is interesting to note that Kuschner[36] reported a failure to induce tumors in hamsters after intratracheal instillations of BP without derric oxide; however, instillations of methylcolanthrene without such a carrier induced a large number of tumors. He attributed these differences to dose, since the rate of disappearance of BP from the lung was more rapid than that of methylcolanthrene in his experimental conditions. However, the experimental details are so limited that it is difficult to evaluate the role of other factors, such as solubility of particle size. Much work remains to be done to clarify the role of vehicles and carriers in the mechanism of action of respiratory carcinogens.

SYSTEMIC ADMINISTRATION OF NITROSAMINES

A variety of carcinogens have been shown to induce respiratory tumors in different species of animals when administered through a systemic route.[2,43]

The systemic respiratory carcinogen most extensively studied in our laboratory has been DEN.[8,9,10,35] We observed that the carcinogenic response of adult and newborn Syrian golden hamsters to DEN follows distinct patterns for the different segments of the respiratory system. The carcinogenicity of DEN in various animal species has been recently reviewed.[8]

Adult hamsters received 12 weekly subcutaneous injections of DEN, each at doses of 4.0, 2.0, 1.0, and 0.5 mg.[8] The results clearly demonstrate a positive dose response correlation for tumor induction in the upper respiratory tract

(nasal cavities, larynx, and trachea). The neoplastic response in the lower respiratory tract remained very low in all four experimental groups.

Newborn hamsters received single subcutaneous injections of DEN at dose levels of 150, 90, 30, and 15 μg.[10] A marked carcinogenic effect on the respiratory tract was observed, with tumor incidences varying from 30% to 65%. As in the adults, the trachea, larynx, and nasal cavities developed the highest tumor yield, whereas the neoplastic response in the lower respiratory tract was low in all the groups.[10] The topography and histology of the induced tumors showed similar patterns in both adult and newborn hamsters. However, it was interesting to find two small cell anaplastic carcinomas in hamsters treated at birth, since no such tumors had ever been induced experimentally before.

The most conspicuous response takes place in the larynx and trachea with the growth of multiple papillary tumors which often occupy the whole lumen, causing the death of the animal by respiratory occlusion. These tumors are lined either by cuboidal mucus-secreting cells or by squamous cells often present in the same tumors, which cannot, therefore, be neatly distinguished into polyps and papillomas, although some individual tumors are found that show only one type of differentiation.[8] It is often practically impossible to count all the individual papillary tumors in the larynx or trachea when their lumen is filled by them. Therefore, we decided that, for the purposes of this presentation, all the papillary tumors of one organ (larynx or trachea) for each animal would be counted cumulatively as one, without distinction of differentiation. Thus we essentially counted tumor-bearing animals, rather than individual tumors, for the laryngeal and the tracheal sites.

Table 3, which is based on such criteria, reports a cumulative analysis of all the respiratory tumors and lesions induced in our studies so far by subcutaneous DEN treatment in adult or newborn hamsters. Of the 405 total tumors thus counted, 96% were localized in the upper respiratory tract (nasal cavities, larynx, and trachea) and only 4% were in the lower respiratory tract. The morphology of the laryngeal and tracheal tumors has just been mentioned. The nasal cavities showed a variety of tumors: squamous cell papillomas and carcinomas, anaplastic carcinomas, adenocarcinomas, and neuroepitheliomas; Table 3 does not attempt a separate classification for each of these types. The bronchi developed a total of only 12 tumors (8 polyps and 3 squamous cell papillomas were all found in the stem bronchi and appeared similar to those of the trachea; only 1 tumor, an adenocarcinoma, appeared to originate from a segmental bronchus.[8] Bronchioles and alveoli showed a very low neoplastic response (1.5%).

Dontenwill *et al.*[21,22] reported a higher incidence of lung tumors in hamsters treated with DEN than we found in our experiments; however, Dontenwill[44] recently repeated those experiments in a different laboratory and observed much lower incidences comparable to those observed by us. Whether tumors of the lower respiratory tract would have developed later, had the animals not died so early from the obstruction of the tracheal lumen, is

TABLE 3

*Respiratory Tract Tumors and Lesions in Hamsters Following Subcutaneous Injection of Diethylnitrosamine**

	Adult	Newborn	Total
Age at time of treatment			
Number of animals autopsied	143	144	287
Number of tumor-bearing animals	135	81	216
Total number of tumors	290	115	405

Sites	No. of tumors†	Types of lesions			
Nasal cavities	94 (23.2%)	Squamous metaplasia, alone	19		19
		Papilloma			
		Carcinoma: Squamous cell, Anaplastic, Adenocarcinoma, Neuroepithelioma	84	10	94
Larynx‡	94 (23.2%)	Squamous metaplasia, alone	2		2
		Papilloma, squamous cell, polyp	67	27	94
Trachea‡	199 (49.1%)	Squamous metaplasia, alone	11		11
		Papilloma, squamous cell, polyp	130	69	199
Bronchi	12 (2.9%)	Squamous metaplasia, alone			
		Papilloma, squamous cell	3		3
		Polyp	3	5	8
		Carcinoma: Squamous cell, Anaplastic			
		Adenocarcinoma	1		1
Bronchioles and alveoli	6 (1.5%)	Squamous metaplasia, alone	2		3
		Adenomatoid lesion	4	4	8
		Carcinoma: Squamous cell, Anaplastic		2	2
		Adenocarcinoma	1		1
		Adenoma	1	2	3

*Cumulative analysis of results pooled from experiments in Syrian golden hamsters of both sexes treated with subcutaneous injections of diethylnitrosamine at different dose schedules

†Number of tumors at each site and their percent out of total number of tumors.

‡Multiple papillary tumors counted as one

obviously difficult to assess. In this regard, we subsequently administered the highest tolerated dose of DEN to adult hamsters and observed a higher incidence of tumors in the lower respiratory tract than in the previous experiments, however, the susceptibility of the bronchi, bronchioles, and alveoli remained strikingly lower than that of the upper respiratory tract [4,5]

Other nitrosamines tested in our laboratory revealed a selective carcinogenic effect for the respiratory tract. Nitrosoazetidine, nitrosoheptamethyleneimine, and nitrosooctamethyleneimine administered orally to rats gave rise to high incidences of respiratory tumors as well as to other types of tumors [5,6] Nitrosoheptamethyleneimine gave a high yield of squamous cell carcinomas in rats. It was subsequently tested in hamsters using the same dose level and route of administration: a number of respiratory tumors were obtained, and, in

contrast to rats, the hamsters also developed squamous cell papillomas and carcinomas of the forestomach.[45]

These results are still being examined.

COMBINED ADMINISTRATIONS OF DEN, BP, AND FERRIC OXIDE

A few experimental reports in the last few years indicated that some additive or synergistic effects on tumor induction may occur in respiratory tract carcinogenesis.[32,33,46–50] One of the most interesting possibilities for synergism in respiratory carcinogenesis is that of the combined effects of topical polynuclear hydrocarbons with systemic carcinogens, such as nitrosamines. The groups of experiments previously described gave us a clear picture of the carcinogenic response of the hamster respiratory tract after separate treatments with BP and ferric oxide and with DEN, and allowed us to approach this intriguing problem.

Two series of experiments were performed using DEN treatment in adult and newborn hamsters as described above, followed by intratracheal administration of BP and ferric oxide. Two groups of adult hamsters received DEN alone at doses which induced only a few tumors of the lower respiratory tract (3 tumors out of a total of 71 animals). Another group received only a suspension of BP with ferric oxide by intratracheal instillations at a subeffective level: 8 animals out of 35 developed a total of 4 tumors in the larynx, 5 in the trachea, and only 1 in the bronchi. Hamsters treated intratracheally with ferric oxide alone did not develop any respiratory tumors (Table 2). Three groups received the combined treatments: when DEN injections were followed, 5 weeks later, by intratracheal instillations of BP + ferric oxide, a remarkable carcinogenic response of the bronchi and lungs, reaching tumor incidences up to 69%, was observed at a relatively early experimental time. Another even more unexpected result was obtained in the group treated with DEN followed by ferric oxide alone: the synergistic carcinogenic response remained very high, with 72% of the animals developing tumors of the lower respiratory tract.[9]

Similar results were obtained by single DEN treatment of newborn hamsters, followed at 10 weeks of age by a course of intratracheal instillations of BP + ferric oxide.[35] In this case we also observed a marked synergistic effect in the induction of tumors in the lower respiratory tract, with incidences of up to 42%. The groups receiving the systemic DEN or the BP + ferric oxide treatments separately showed tumor incidences of 9% and 8%, respectively. Although most of the tumors occurred in the bronchiolo-alveolar region and appeared adenomatous, some did show a direct origin from the epithelium of segmental bronchi; the synergistic effect therefore involves the whole broncho-pulmonary system. The morphology of these tumors is described elsewhere.[9,35]

These findings demonstrate that a preliminary systemic treatment of the hamsters with a relatively low dose of a tissue-specific carcinogen, such as DEN, prepares them to respond with a high degree of susceptibility not only to a level

of BP, which by itself only appeared to cause a weak response, but even to an inorganic particulate material, such as ferric oxide, which had been extensively tested by itself with negative results.

CONCLUSIONS

The present observations demonstrate that different carcinogenic agents alone or in combination affect the various segments of the respiratory tree with distinct patterns and elicit morphologically different types of lesions. The response is specifically related to the animal species and to the type of treatment.[2,15,51] For instance, tumors of the trachea were frequently induced in hamsters by intratracheal administrations of polynuclear hydrocarbons and by systemic administrations of DEN and other nitrosamines, but tracheal tumors were rarely reported in rats and not at all in mice; it appears that the tracheal mucosa is more susceptible to carcinogens in hamsters than in mice and rats.[52] On the other hand, rats are more prone to develop tumors of the bronchiolo-alveolar region in response to a variety of agents, ranging from polynuclear hydrocarbons and nitrosamines to radioactive particles and inorganic materials such as beryllium sulphate,[53] nickel carbonyl,[54] and asbestos.[23] The susceptibility of mice to lung adenoma induction by various agents is, of course, well known.

Bronchogenic squamous cell carcinomas could be induced in animals only in certain experimental conditions: high incidences were obtained by intratracheal instillations of BP in hamsters and in rats, using the methodology developed in our laboratories[11,12] as well as other methods,[28,39,40,42,55] or by implantation in the bronchial lumen of pellets containing polynuclear hydrocarbons or radioactive material.[36–38,56] DEN administered systemically only induced some papillomatous tumors in the stem bronchi.

In this regard we should like to mention some preliminary results of a series of experiments (analogous to those described above with synergistic effects in adult hamsters) in which the sequence of treatments was reversed. The intratracheal instillations of mixed BP + ferric oxide or of ferric oxide alone were administered *before* the DEN injections. Ferric oxide alone, followed by DEN, in this case does not produce any synergistic or additive effects in the induction of lung tumors, but results in a tumor incidence similar to that found in the control DEN-treated hamsters. On the other hand, when BP is added to ferric oxide, their mixed suspension given intratracheally and then followed by DEN, the hamsters developed higher incidences of squamous cell carcinomas of the trachea and stem bronchi than in the respective controls. These findings suggest that the conditions required for ferric oxide to determine a synergistic effect in the induction of broncho-pulmonary tumors exist only when ferric oxide is administered *after* DEN and do not function when it is given before DEN. We suggest that the mechanism of such effect is linked to the cellular reaction to the instillation of the particulate material. Furthermore, DEN

administered *after* the instillations of BP mixed with ferric oxide increased the number of squamous cell carcinomas at the sites that have been shown to be particularly susceptible to the carcinogenic action of higher doses of BP. Each of the two carcinogens seems capable of stimulating the development of tumor types characteristic of the direct effect of the other.

These results show that, by selecting an appropriate experimental model, we can now reproduce a whole spectrum of tumor responses in the different segments of the respiratory tract, from the nasal cavities down to the alveoli, and correlate them with the specific activity of various carcinogens. We can now approach the next phase of the investigations: an attack on the problem of identifying the key factors that condition each type of response, such as the metabolic aspects of the fate of carcinogens in the lungs.

In the light of the reported observations, the mechanism of induction of respiratory tumors by polynuclear hydrocarbons carried by particles needs to be further analyzed; the role of particulates used as carriers in other experimental systems – such as gelatin,[7] casein, and India ink powder[39,40,57] – should be further assessed. The physicochemical factors required for activity can be analyzed by using the existing experimental models.

The present studies are basic to the understanding of the pathogenesis of lung cancer and have a direct bearing on our ability to evaluate human carcinogenic hazards. Of the three main agents investigated, BP is a well-known environmental contaminant,[1,4,32,34,39,40,50,51,54,58] DEN is representative of the class of nitrosamines that occur environmentally,[59,60] and ferric oxide also represents a common environmental contaminant[61] that has been suggested as a possible human lung cancer risk.[61] Much remains to be learned about the interplay of a variety of etiologic factors in the causation of lung cancer; our recent acquisitions may contribute a new insight into this challenging problem.

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REFERENCES

1. L. Severi (ed.), *Lung Tumours in Animals, Proc. 3rd Quadrennial Intern. Conf. Cancer.* Div. of Cancer Res., Univ. of Perugia, Italy, 1966.

2 U Saffiotti, Experimental respiratory tract carcinogenesis In, *Progress in Experimental Tumor Research* Vol 11, pp 302 333, F Homburger (ed) Karger New York/Basel, 1969

3 U Safiotti, Experimental respiratory tract carcinogenesis and its relation to inhalation exposures In, *Inhalation Carcinogenesis* proceedings of a conference held in Gatlinburg Tennessee, October 8–11, 1969, AEC Symposium Series, No 18 (CONF 691001), pp 27–54, M G Hanna, Jr , P Nettesheim, J R Gilbert (eds), 1970

4 P Shubik, G Della Porta, G Pietra, L Tomatis, H Rappaport U Saffiotti, and B Toth, Factors determining the neoplastic response induced by carcinogens In, *Biological Interactions in Normal and Neoplastic Growth,* pp 285–297, M J Brennan and W L Simpson (eds) Little, Brown and Co Boston, 1962

5 W Lijinsky, K Y Lee, L Tomatis, and W H Butler Nitroso-azetidine – A potent carcinogen of low toxicity *Naturwissenschaften,* 54 (1967) 518

6 W Lijinsky, L Tomatis and C E M Wenyon, Lung tumors in rats treated with N-nitrosoheptamethyleneimine and N-nitrosooctamethyleneimine *Proc Soc Exptl Biol and Med* , 130 (1968) 945–949

7 G Della Porta, L Kolb, and P Shubik, Induction of tracheobronchial carcinomas in the Syrian golden hamster *Cancer Res* 18 (1958) 592–597

8 R Montesano and U Saffiotti, Carcinogenic response of the respiratory tract of Syrian golden hamsters to different doses of diethylnitrosamine *Cancer Res* 28 (1968) 2197–2210

9 R Montesano, U Saffiotti, and P Shubik, Synergistic effects of diethylnitrosamine, benzo[*a*] pyrene and ferric oxide on respiratory carcinogenesis in hamsters *Cancer Res* in press

10 R Montesano, U Saffiotti, and P Shubik, Carcinogenic response of hamster respiratory tract to single subcutaneous administrations of diethylnitrosamine at birth *J Natl Cancer Inst* 44 (1970) 413 417

11 U Saffiotti, S A Borg, M I Grote, and D A Karp, Retention rates of particulate carcinogens in the lungs Studies in an experimental model for lung cancer induction *The Chicago Medical School Quart* , 24, No 2, (1964) 10–17

12 U Saffiotti, F Cefis, L Kolb, A method for the experimental induction of bronchogenic carcinoma *Cancer Res* 28 (1968) 104–124

13 U Saffiotti, F Cefis, L H Kolb, and P Shubik, Experimental studies on the conditions of exposure to carcinogens for lung cancer induction *J Air Pollution Control Assoc* 15 (1965) 23–25

14 U Saffiotti, F Cefis, and P Shubik, Histopathology and histogenesis of lung cancer induced in hamsters by carcinogens carried by dust particles In, *Lung Tumours in Animals, Proc 3rd Quadrennial Intern Conf Cancer* pp 537–546, L Severi (ed) Div of Cancer Res , Univ of Perugia, Italy, 1966

15 U Saffiotti, R Montesano, and A R Sellakumar, Correlation between the histogenesis of respiratory tract tumors and their mechanisms of induction *Am J Pathol* 52 (1968) 59a–60a

16 U Safiotti R Montesano, and A R Sellakumar, unpublished observation

17 U Saffiotti, R Montesano, A R Sellakumar, and S A Borg, Studies on experimental lung cancer Inhibition by vitamin A of the induction of tracheobronchial squamous metaplasia and squamous cell tumors *Cancer,* 20 (1967) 857–864

18 U Saffiotti, R Montesano, A R Sellakumar, and F Cefis, Respiratory tract carcinogenesis in hamsters induced by different numbers of administrations of benzo[*a*] pyrene and ferric oxide *Cancer Res* in press

19 U Saffiotti, R Montesano, and N Tompkins, Benzo[*a*] pyrene retention in hamster

lungs: Studies on particle size and on total dust load. *Proc. Am. Assoc. Cancer Res.* 8 (1967) 57.

20. W. Dontenwill, Vergleichende Untersunchungen uber die Organotrope Wirkung Verschiedener Nitrosamine. In, *Alkylierend Wirkende Verbindungen, Erste Konferenz uber N-Nitroso-Verbindungen und Lactone,* pp. 67–74, Wissenschaftliche Forschungsstelle im Verband der Cigarettenindustrie. Hamburg, Germany, 1964.
21. W. Dontenwill and U. Mohr, Carcinome des Respirationstractus nach Behandlung von Goldhamstern mit Diathylnitrosamin. *Z. Krebsforsch.,* 64 (1961) 305–312.
22. W. Dontenwill, U. Mohr, and M. Zagel, Uber die Unterschiedliche Lungencarcinogene Wirkung des Diathylnitrosamin bei Hamster und Ratte. *Z. Krebsforsch.,* 64 (1962) 499–502.
23. P. Gross, R. T. P. De Treville, E. B. Tolker, M. Kaschak, and M. A. Babyak, Experimental asbestosis – The development of lung cancer in rats with pulmonary deposits of chrysotile asbestos dust. *Arch. Environ. Health,* 15 (1967) 343–355.
24. P. Gross, E. Tolker, M. A. Babyak, and M. Kaschak, Experimental lung cancer in hamsters. *Arch. Environ. Health,* 11 (1965) 59–65.
25. K. M. Herrold, Induction of olfactory neuroepithelial tumors in Syrian hamsters by diethylnitrosamine. *Cancer,* 17 (1964) 114–121.
26. K. M. Herrold, Epithelial papillomas of the nasal cavity, *Arch. Pathol.,* 78 (1964) 189–195.
27. K. M. Herrold, Effect of route of administration on the carcinogenic action of diethylnitrosamine (*N*-nitrosodiethylamine). *Brit. J. Cancer,* 18 (1964) 763–767.
28. K. M. Herrold and L. J. Dunham, Induction of carcinoma and papilloma of the tracheobronchial mucosa in the Syrian hamsters by intratracheal instillations of benzo[*a*]pyrene. *J. Natl. Cancer Inst.,* 28 (1962) 467–491.
29. K. M. Herrold and L. J. Dunham, Induction of Tumors in the Syrian hamster with diethylnitrosamine (*N*-nitrosodiethylamine). *Cancer Res.,* 23 (1963) 773–777.
30. U. Mohr, J. Althoff, and A. Authaler, Diaplacental effect of the carcinogen diethylnitrosamine in the golden hamster. *Cancer Res.* 26 (1966) 2349–2352.
31. U. Mohr, O Wieser, and K. Pielsticker, Die minimaldosis fur die Wirkung von Diathylnitrosamine auf die Trachea beim Boldhamster. *Naturwissenschaften,* 53 (1966) 229.
32. E. L. Wynder and D. Hoffmann, Biological tests for tumorigenic and cilia-toxic activity. In, *Tobacco and Tobacco Smoke. Studies in Experimental Carcinogenesis,* pp. 179–315, E. L. Wynder and D. Hoffmann (eds.). Academic Press, New York, 1967.
33. E. L. Wynder, K. Taguchi, V. Baden, and D. Hoffmann, Effect of passive inhalation of cigarette smoke on the respiratory tract of mice and hamsters. *Proc. Am. Assoc. Cancer Res.,* 7 (1966) 77.
34. B. Toth, L. Tomatis, and P. Shubik, Multipotential carcinogenesis with urethan in the Syrian golden hamster. *Cancer Res.,* 21 (1961) 1537–1541.
35. R. Montesano and U. Saffiotti, unpublished observation.
36. M. Kuschner, The J. Burns Amberson lecture – The causes of lung cancer, *Am. Rev. Respirat. Diseases,* 98 (1968) 573–590.
37. M. Kuschner, S. Laskin, E. Cristofano, and N. Nelson, Experimental carcinoma of the lung. In, *Proc. 3rd Natl. Cancer Conf.* pp. 485–495. Lippincott, Philadelphia, 1956.
38. M. Kuschner, S. Laskin, M. Daniels, and N. Nelson, The pathogenesis of radiation induced bronchogenic carcinoma in rats. In *Lung Tumours in Animals, Proc. 3rd Quadrennial Intern. Conf. Cancer,* pp. 369–377, L. Severi (ed.). Div. of Cancer Res., Univ. of Perugia, Italy, 1966.
39. L. M. Shabad, Pathology and pathogenesis of experimental lung tumours with reference to human lung cancer prevention. In, *Lung Tumours in Animals, Proc. 3rd Quadrennial*

Intern. Conf. Cancer, pp. 85–100, L. Severi (ed.). Div. of Cancer Res., Univ. of Perugia, Italy, 1966.

40. L. M. Shabad, Experimental cancer of the lung. *J. Natl. Cancer Inst.*, 28 (1962) 1305–1332.
41. W. J. Clarke, F. J. Park, and W. J. Bair, Plutonium particle-induced neoplasia of the canine lung. II. Histopathology and conclusions. In, *Lung Tumours in Animals, Proc. 3rd Quadrennial Intern. Conf. Cancer*, pp. 345–355, L. Severi (ed.). Div. of Cancer Res., Univ. of Perugia, Italy, 1966.
42. L. N. Pylev, Experimental induction of lung cancer in rats with 3,4-benzpyrene (in Russian). *Vestn. Akad. Med. Nauk, SSSR*, 19 (1964) 41–45.
43. R. Kinosita, Experimental lung tumors in animals. In, *Lung Tumours in Animals, Proc. 3rd Quadrennial Intern. Conf. Cancer*, pp. 59–84, L. Severi (ed.). Div. of Cancer Res., Univ. of Perugia, Italy, 1966.
44. W. Dontenwill, personal communication.
45. R. Montesano *et al.*, unpublished observation.
46. W. Dontenwill, Experimentelle Untersuchungen zur Genese des Lungen-carcinoms. *Arzneimittel Forsch.*, 14 (1964) 774–780.
47. C. Hoch-Ligeti, M. F. Argus, and J. C. Arcos, Combined carcinogenic effects of dimethylnitrosamine and 3-methylcholanthrene in the rat. *J. Natl. Cancer Inst.*, 40 (1968) 535–549.
48. P. Kotin and D. V. Wiseley, Production of lung cancer in mice by inhalation exposure to influenza virus and aerosols of hydrocarbons. *Progr. Exptl. Tumor Res.*. 3 (1963) 186–215.
49. K. Mori, Acceleration of experimental lung cancer in rats by inhalation of cigarette smoke. *Gann*, 55 (1964) 175–181.
50. M. Toda, Experimental studies of occupational lung cancer. *Bull. Tokyo Med. Dent. U.*, 9 (1963) 440.
51. H. P. Stewart, Comparison of histologic lung cancer types in captive wild mammals and birds and laboratory and domestic animals. In, *Lung Tumours in Animals, Proc. 3rd Quadrennial Intern. Conf. Cancer*, pp. 25–58, L. Severi (ed.). Div. of Cancer Res., Univ. of Perugia, Italy, 1966.
52. W. Dontenwill and B. Wiebecke, Tracheal and pulmonary alterations following the inhalation of cigarette smoke by the golden hamster. In, *Lung Tumours in Animals, Proc. 3rd Quadrennial Intern. Conf. Cancer*, pp. 519–525, see discussion pp. 525–526, L. Severi (ed.). Div. of Cancer Res., Univ. of Perugia, Italy, 1966.
53. A. L. Reeves, D. Deitch, and A. J. Vorwald, Beryllium carcinogenesis. I. Inhalation exposure of rats to beryllium sulfate aerosol. *Cancer Res.*, 27 (1967) 439–445.
54. F. W. Sunderman, Metastasizing pulmonary tumors in rats induced by the inhalation of nickel carbonyl. In, *Lung Tumours in Animals, Proc. 3rd Quadrennial Intern. Conf. Cancer*, pp. 551–564, L. Severi (ed.). Div. of Cancer Res., Univ. of Perugia, Italy, 1966.
55. L. N. Pylev, Induction of lung cancer in rats by intratracheal insufflation of cancerogenic hydrocarbons. *Acta Unio Intern. Contra Cancer*, 19 (1962) 688–691.
56. S. Laskin, M. Kuschner, N. Nelson, B. Altshuler, J. H. Harley, and M. Daniels, Carcinoma of the lung in rats exposed to the β-radiation of intrabronchial ruthenium^{106} pellets. I. Dose-response relationships. *J. Natl. Cancer Inst.*, 31 (1963), 219–231.
57. L. N. Pylev, F. J. C. Roe, G. P. Warwick, Elimination of radioactivity after intratracheal instillation of tritiated 3,4-benzopyrene in hamsters. *Brit. J. Cancer*, 23 (1969) 103–115.
58. E. Sawicki, W. C. Elbert, R. Thomas, M. S. Hauser, F. T. Fox, and T. W. Stanley, Benzo[*a*] pyrene content of the air of American communities. *Am. Indust. Hyg. Assoc. J.*, 21 (1960) 443–451.

59 L S DuPlessis, J R Nunn, and W A Roach, Carcinogen in Transkeian Bantu food additive *Nature* 222 (1969) 1198–1199
60 P N Magee and J M Barnes, Carcinogenic nitroso compounds In, *Advances in Cancer Research* Vol 10, pp 163–246, A Haddow and S Weinhouse (eds) Academic Press, New York, 1967
61 W C Hueper, *Occupational and Environmental Cancers of the Respiratory System* p 212 Springer Verlag, New York, 1966

DISCUSSION

E. P. Radford: Since some of us will not have the benefit of hearing the discussions at the May meeting [Conference on Morphology of Experimental Respiratory Carcinogenesis, May 13–16, 1970, Gatlinburg, Tenn], where morphology of the type just presented will presumably be discussed in detail, I wonder if the morphologists here will clear up a couple of quite basic points on lung cancer for the rest of us who are not morphologists First, is it a fact that respiratory squamous cell carcinomas must arise from bronchial epithelium? Second, isn't the site of origin of squamous cell carcinoma in the bronchial tree dependent on local doses of carcinogens or cocarcinogens at these sites?

R. Montesano: The squamos tumors do not necessarily arise from the mucosa of the lung bronchi Some squamous tumors also arise from the bronchiolar alveolar area, as reported in different animal species

The type of induced tumors is also related to the type of treatment Using the technique of intratracheal instillations of benzpyrene carried by ferric oxide, the area around the bifurcation of the trachea develops the highest number of squamous tumors, while after diethylnitrosamine most of the tumors are benign polyps of the trachea, showing in some instances a squamous differentiation.

T. Crocker: I think what you have said is that you are able to recognize squamous tumors arising from airways close to the alveolar end of the respiratory tract, but that these are still rising in columnar epithelial cells Is that correct?

Montesano: Where the peripheral tumors arise is difficult to say In these series of experiments we did not do any histogenic studies These peripheral tumors could arise either from the terminal bronchioles or the alveoli.

Crocker: Perhaps the question of the cell type from which squamous cells arise is not completely answered At the moment it seems as though the squamous tumors in hamsters, referred to by Dr Montesano, are more likely to be alterations of cells previously of the columnar type, whether they are in the smaller bronchi or in the larger airways Would Dr Montesano agree with this, or would you like to make further comment?

M. Kuschner: I assume that Dr Radford is referring to human tumors, now.

Radford: Or in the experimental situation

Kuschner. Well, I think in the experimental situation you have to admit that there is such a thing as squamous metaplasia of the alveolar lining, and that this metaplasia in turn can give rise to squamous carcinoma I think this situation does not apply in most human instances, however The particular value of Dr Auerbach's work is that he demonstrated that there is a substrate, a change in the bronchi which involves atypical hyperplasia and atypical metaplasia, in which most human tumors arise, and I think that we are concerned for the most part with an origin from previously altered bronchial epithelium in the human tumor

As to the reason for this, you ask whether this is purely a dosage phenomenon, and I think that local dosage is indeed one major determinant of where the tumor arises

However, since we feel that there are multiple factors involved in this transformation, and even in preparing the substrate on which the tumor develops, I think that besides dosage we have to concern ourselves with other things that determine the particular susceptibility or sensitivity of the bronchial epithelium in the human situation

J. Kleinerman: I think that before we leave the problem of human tumors it should be mentioned that many human bronchial tumors have elements of both squamous and adenomatous neoplastic cells I think that this tends to confuse the issue of the cell of origin and makes it extremely difficult for the pathologist to say whether the tumor cells are exclusively of squamous or adenomatous origin. It may be that the basal cell is the cell of origin from which both of these more mature elements are derived

F. J. C. Roe: It could be argued that the lung of a small animal is anatomically quite different from the human lung. It is very difficult to know the proportions of cancers that arise from central and peripheral locations in man, but even where a peripheral origin seems most likely, the site of origin may be in an anatomical structure that is simply not present in the small rodent lung. It could be argued that the trachea and lungs of the mouse are equivalent to no more than a peripheral segment of the human lung. In the mouse only the trachea and main bronchi have cartilage, and it is generally stated that there are no bronchial mucous glands.

I know of one instance where a so-called squamous carcinoma of the lung in a mouse grew as an adenocarcinoma upon transplantation This, and other observations, lead me to doubt whether such tumors in mice should be regarded as models for squamous lung cancer in man

Montesano: The squamous tumor induced in mice is not comparable to the human squamous tumor, but in hamsters or rats, with the technique of Saffiotti or the technique of the pellet implantation of Laskin and Kuschner, it is possible to reproduce a bronchogenic squamous tumor that is very similar to the human counterpart The sophism concerning the similarity between the anatomy of the

large bronchi of rats or hamsters and those of the humans, from my point of view, is not so important. So far we have a reproducible model closely simulating the human counterpart. I think it is better to stick with it and try to understand the pathogenesis of the induced tumors. I don't think you can extrapolate the results obtained with the mice to the human situation.

Crocker: I think Dr. Roe's point is a good one; that is, it is desirable to establish the comparability of the squamous changes in the human and hamster tumors, whether squamous carcinoma or squamous metaplasia. There are, by most criteria, very good parallels. But I think Dr. Roe's additional point is an important one, and that is that the early lack of glands in the peripheral airways of the rodent lung reduces the probability of adenocarcinomas of glandular origin, which may occur early in the human lung but may originate in glands in the peripheral bronchi and may not be of alveolar cell origin.

U. Saffiotti: A couple of points. First, I would like for Dr. Nettesheim to comment on the presence of glands in the bronchial tract of rodents; I think he has some pertinent data on that. Second, I think we should consider what Marvin Kuschner has pointed out before, that we are studying the induction of tumors in a given tissue and not so much in a whole organ, whether the small lung of the mouse or the big lung of man. I think the important factor, which we have been trying to define better in these models, is the induction of certain cellular reactions that are similar, at the cellular level, to those that we see in man. The fact that some of the tumors indeed show different types of differentiation in the same tumor indicates a variability in the equilibrium of various types of differentiation. We have found this balance in a good proportion of our cases. The results of our studies on the role of vitamin A, which Dr. Crocker has also obtained in his *in vitro* system, indicate that this balance of differentiation could possibly be controlled by special factors related to the development of the neoplastic changes.

I think this is the key – not so much the anatomical but rather the tissue characterization and the cellular differentiation.

P. Nettesheim: As to whether or not small rodents have tracheobronchial glands, we found in our mouse studies, to our great surprise, that in spite of a lot of reports to the contrary, mice do have tracheobronchial glands. To be more specific, glands develop in the lower portion of the trachea and in the major bronchi with advancing age. If you look at groups of 7- to 10-week-old mice (and this includes four or five strains that we have looked at), you find practically no such glands. When you look at a 1- to 2-year-old mouse, however, you find an abundance of glands, not only in the lower portion of the trachea but also in the first and second generation of bronchi.

Crocker: Thank you. I think it is important to emphasize an age-related acquisition of glandular structures in rodents. They are not there at all in the suckling or newborn animal.

Kuschner: I think that many of us (and I know I, particularly) had felt that the use of systemic urethane in the induction of mouse adenomas was not at all pertinent – that it was totally irrelevant to the human tumor induction problem. Yet here we have a very clear example of the introduction of what might be called a systemic initiator or systemic carcinogen and then its effect made manifest by treatment in a particular site, the lung. I don't think we've ever had that kind of demonstration before, that a bronchogenic carcinoma could indeed appear in the presence of systemic initiation. Here we have the kind of situation where one gives a systemic medium, or material, and by additional treatment produces a tumor in the site that we are concerned with. One might even be willing to speculate that the prevalence of tracheal tumors, in the instances where diethylnitrosamine is used alone, might be related to the observation reported to us earlier by Dr. Kleinerman. He pointed out that in the hamster the replication rate is greatest in the trachea, under normal conditions. This might be the situation that permits expression of the effect of diethylnitrosamine in the absence of other treatment. But when one alters the response of the lung by giving a material that does affect the bronchi in the presence of this systemic material, one gets a tumor in that area. I think this is a consideration that none of us had entertained before in our thinking about lung cancer, and certainly it deserves high marks.

J. R. Prine: In regard to utilizing older laboratory animals, especially rats, I would like to caution all of you to be sure, in doing a complete necropsy, to examine the bulla tympanicum, because here you may find evidence of chronic rodent pneumonia that is adversely influencing your experiment.

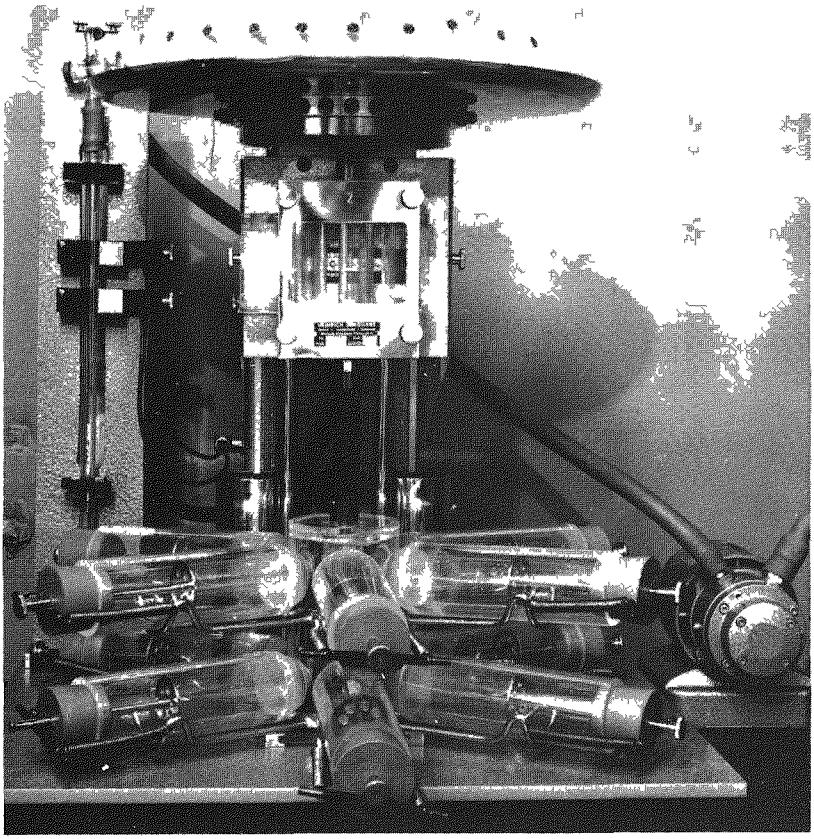
SESSION IV

CURRENT STUDIES IN INHALATION CARCINOGENESIS

Current Studies with Multiple Treatment

Special Topics

Chairman – Norton Nelson
Institute of Environmental Medicine
New York University Medical Center, New York



EMPHYSEMA PRODUCED IN DOGS BY CIGARETTE SMOKING

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ABSTRACT

Ten dogs smoked cigarettes daily in two sessions per day by voluntary inhalation through a tracheostomy tube. Ten additional dogs were kept as controls. Five dogs died during the course of the experiment and the remaining five were sacrificed after 420 days of cigarette smoking. The ten control dogs were also sacrificed at this time. — Hematocrit and percent hemoglobin levels increased markedly during the first several weeks of smoking; then they declined somewhat but still remained higher than presmoking levels. Heart weight relative to body weight was markedly higher in the ten smoking dogs than in the ten controls. Pulmonary fibrosis and emphysema, similar to that seen in human beings, were found in all five of the sacrificed smoking dogs. No such lung parenchymal changes were found in the ten controls.

This study was initially undertaken simply as a preliminary experiment to determine the maximum number of cigarettes that dogs can smoke daily without showing severe acute effects of nicotine or carbon monoxide poisoning. We were not interested in dosage levels, relative to body weight, much beyond those found in some extremely heavy human cigarette smokers. When it was found that dogs could tolerate a fairly high exposure, the experiment was continued for over a year to get some idea of the long-term effects. When two dogs died with such massive lung infarction that the tissue could not be evaluated for structural changes, the remaining dogs were sacrificed. Causes of death, lung parenchymal changes, hematocrit and hemoglobin changes, and heart weights will be discussed here. A microscopic study of the bronchial tubes of the dogs has also been reported.[1]

METHOD

Tracheostomy was performed on each dog and 3 weeks were allowed for recovery. The tracheostomy was kept open with a specially designed hollow tube made of Teflon. In smoking, this tube was replaced by a Teflon tube equipped with a socket for coupling to a machine designed to deliver cigarette smoke intermittently.[2] The first one or two puffs (to light the cigarette) were delivered under forced draft. Thereafter, the cigarette was smoked by deep voluntary inhalation of the dog. Between each five puffs (or more often when necessary to prevent anoxia), the smoking tube was clamped off so that the dog could breathe fresh air through its mouth or nostrils. Whenever a dog showed signs of distress, a longer period of air inspiration was permitted.

Initially, the dogs showed high excitability and symptoms such as those occurring in a child attempting to smoke his first cigarette (slight to profuse salivation, coughing, dilatation of pupils, redness and tearing of the eyes, and, sometimes, nausea, vomiting, and dizziness). These symptoms subsided substantially in subsequently days but reappeared from time to time, the dogs being variable in this respect. The dogs were cooperative, and, after a week or two, some showed that they liked cigarette smoking by wagging their tails and jumping into the smoking box voluntarily.

PROCEDURE

The plan was to increase daily cigarette consumption gradually, cutting back to a lower level whenever the reaction of the dogs indicated that we might have exceeded tolerable limits (i.e., when we found acute symptoms more severe than those experienced by beginning human cigarette smokers). One female and nine male beagles of pedigreed stock were selected as experimental animals. They were divided into two groups, A and B, the two lightest dogs being put into Group A and the two heaviest into group B (see Table 1).

On 4 successive days, each dog was put in the smoking apparatus in the morning and again in the afternoon, all procedures being carried out except that the cigarette was not lighted. On the morning of the next day (which we will call day 1), each animal was given one lighted filter-tip cigarette of a brand reported to be low in tar and nicotine content, and on the afternoon of that day was given one unlighted cigarette. This was repeated on day 2. On days 3 to 6 one lighted filter-tip cigarette was given in the morning and one in the afternoon. On days 7 to 10, two cigarettes of a popular 70-mm nonfilter-tip brand were given, one in the morning and one in the afternoon. Cigarettes of this brand (which was reported to be relatively high in nicotine and tar content) were used thereafter. Each cigarette was smoked down to a remaining butt length of approximately 5 mm.

Daily dosage throughout the remainder of the experiment is shown in Table 2. There were two smoking sessions per day, morning and afternoon, 7 days a week. On days when the dogs smoked an odd number of cigarettes, one more

TABLE 1

Age at Start of Smoking, Days from Start to Death, Number of Cigarettes Smoked, Weights, and Mean Hematocrits for Each of the Ten Experimental Dogs

Dog number*	Age at start (months)	Days from start to death†	Number of cigarettes smoked	Weight (lbs)		Mean hematocrit (%)				
				At start	At end	Before‡ smoking	Days 7–11§	Days 16–20§	Highest 2 weeks¶	Last 2 weeks¶
					Group A					
29 M	19	24 D	73	22.5	22.5	45.9	50.2	51.0	51.8	51.8
30 M	19	229 D	1,378	16.8	16.5	47.8	55.7	55.3	65.3	59.4
32 F	25	410 D	3,539	25.5	24.0	49.1	60.1	58.1	67.4	53.4
36 M	30	415 D	3,598	23.5	24.8	46.8	57.9	65.3	68.1	48.2
37 M	9	423 S	3,702	24.5	26.3	49.0	50.5	55.9	59.5	56.6
					Group B					
15 M	21	422 S	4,114	36.0	33.3	49.3	57.0	58.1	66.4	51.4
26 M	9	423 S	4,084	28.3	32.3	48.9	52.1	55.2	64.4	53.7
31 M	17	421 S	4,104	27.8	26.3	48.4	56.9	58.6	69.5	57.7
34 M	18	422 S	4,116	22.5	22.8	47.8	56.1	59.0	66.5	51.8
35 M		278 D	2,298	25.5	25.0	48.5	52.1	55.2	60.8	55.5

*M, male; F, female.

†D, died; S, sacrificed.

‡Mean of readings on 4 successive days before smoking.

§Mean of readings on 5 successive days (smoking cigarettes).

¶Mean of readings taken during a period of 2 weeks.

TABLE 2

Number of Cigarettes Smoked Daily by Dogs in Groups A and B

Day from start of smoking	Number of cigarettes smoked per day	
	Group A	Group B
1–2	1	1
3–10	2	2
11	2	4
12–15	3	4
16–20	4	6
21–23	6	8
24	5	7
25–29	3	5
30	4	6
31–37	5	7
38–106	4	6
107–120	5	7
121–148	6	8
149–162	7	9
163–176	8	10
177–190	9	11
191–204	10	12
205–207	11	13
208–226	10	12
227–423	12	12

cigarette was given in the morning than was given in the afternoon. The dogs were fed once a day, about half an hour after the afternoon session. As shown in Table 2, up until day 227, dogs in group A were given fewer cigarettes per day than dogs in group B.

Several hours after the morning smoking session on day 24, an apparently healthy dog that appeared to have good tolerance for cigarette smoking was found dead in its cage. Fearing that we might have exceeded tolerable limits, we reduced the daily cigarette smoking of the remaining nine dogs for several days. On day 31, cigarette consumption was increased but was reduced again on day 38 due to acute symptons in several dogs. Later, consumption was increased again without appearance of acute symptoms.

It should be noted that Table 2 shows the general schedule of dogs in groups A and B. Occasionally, owing to symptoms, an individual dog was given a temporary reduction in smoking. For example, on two occasions dog 35 was temporarily taken off smoking and given antibiotic therapy for hyperpyrexia, fever, and apparent pulmonary infection. This dog had to be handled carefully – often being given fresh air between puffs of cigarette smoke – and he appeared not to inhale the smoke quite as deeply as most of the other dogs. Dog 31

developed convulsions on day 24. Smoking was immediately reduced but later resumed at the level of the other dogs in group B.

Five of the experimental dogs died during the course of the experiment and the remaining five were sacrificed on days 421, 422, and 423.

Ten additional dogs of the same breed and of approximately the same sizes and ages as the ten smoking dogs were kept as controls. Two of them had tracheostomies. They were not exposed to cigarette smoke but were sacrificed at the same time as the last five experimental dogs.

FOOD INTAKE AND BODY WEIGHT

Food intake and body weight were determined daily. The appetites of the smoking dogs remained excellent, although this varied somewhat from time to time. As indicated in Table 1, none of the dogs showed a marked change in weight up to the time of death or sacrifice.

HEMATOCRIT

Ear blood samples were taken from the experimental dogs on 4 successive days before the start of smoking and once a day immediately following the morning smoking session on days 1 through 26. Thereafter, ear blood was drawn every Tuesday and Friday immediately after the morning smoking session. Ear blood samples were similarly drawn from five of the control dogs on Tuesday and Friday mornings during 27 consecutive days. Of these five dogs, two had tracheostomies and three did not.

Microhematocrits were determined by the standard capillary tube technique.[3]

The mean hematocrit of the five control dogs was 47.7, 47.9, 45.3, 50.7, and 48.7%, respectively. These results are in agreement with a previous report on normal male beagles.[4] Although the hematocrit readings for individual dogs varied from time to time, there was no appreciable trend either up or down during the 27 weeks of observation. The highest single hematocrit reading for any of these five dogs was 55.5%. As shown in Table 1, the mean hematocrits of the ten smoking dogs on the 4 days before smoking were in the range from 45.9 to 49.3%, i.e., in the same range as that of the control dogs. Of the 40 individual readings taken during this period (four readings on each of the 10 dogs), the highest was 51.0%.

The hematocrits of all 10 experimental dogs began to rise when they started to smoke. The mean reading for each of the dogs on days 7 to 11 and on days 16 to 20 is shown in Table 1. For all 10 dogs the mean hematocrit during days 7 to 11 was higher than before smoking, and in 8 of the 10 dogs it was still higher during days 16 to 20 After this, the readings fluctuated considerably but showed a tendency to rise in all 10. The highest mean of any four readings taken consecutively during a 2-week period varied from 51.8% (dog 29) to 69.5% (dog 31). In dog 36, the highest mean level occurred during the 4th and 5th weeks of

smoking, while in most of the other dogs it was not reached until the 12th to 14th week of smoking In later weeks, despite an increase in daily cigarette consumption, the mean hematocrit readings tended to decline, the degree of decline varying in different dogs (note last column in Table 1) The highest single hematocrit reading for each of the ten dogs was dog 29, 57 0%, dog 30, 68.0%, dog 32, 71 0%, dog 36, 69.5%, dog 37, 68 0%, dog 15, 71.0%, dog 26, 67 0%, dog 31, 71 0%, dog 34, 72.0% and dog 35, 64 0%

CAUSES OF DEATH

Dog 29 died unexpectedly on day 24. At autopsy a large recent thrombus was found in the right auricular appendage In addition, several recent infarcts measuring from 5 mm to 2 cm were found in both lungs

Dog 30 died suddenly on day 229 while smoking the fourth cigarette of the afternoon session He had shown signs of ill health during the preceding several days Autopsy indicated recent bronchopneumonia (with no evidence of abscess formation) as the cause of death.

Dog 35 died on day 278 while inhaling smoke from the third cigarette of the afternoon session He had appeared to be in fairly good health during the preceding several weeks At autopsy, thrombi and areas of infarction were found in both lungs.

Dog 32 was found dead early on the morning of day 410. She had had wheezy, labored respiration for several weeks, her movements were slow, and she appeared to be fatigued Frequent rectal temperatures taken during this period were normal At autopsy a recent thrombus was found in the right auricular appendage, and emboli with massive infarction were found in both lungs.

Dog 36 was found dead on the morning of day 415. The animal had been ill for several days with a high fever and was under treatment with antibiotics and intravenous fluids At autopsy a large recent thrombus was found in the right auricular appendage, and there was massive infarction of both lungs as well as secondary pneumonia

FINDINGS IN LUNG PARENCHYMA

Soon after their removal from the body, all the lungs were filled with formalin instilled into the trachea A specimen of parenchymal tissue was taken from each lobe for microscopic study (the lungs of beagles have seven lobes).

Grossly, the lungs of all 10 control dogs showed a pink surface, as did the cross sections of the lungs. Microscopically, the lung parenchyma of these dogs showed a uniform appearance that was strikingly similar to that of a human being who has never smoked or been exposed to air pollutants (Figs. 1b, d and 2b, d).[5] The size of the alveolar spaces were within normal limits, and the alveolar septa were thin. In none of the 10 control dogs was there evidence of pulmonary fibrosis or rupturing and tearing of alveolar septa.

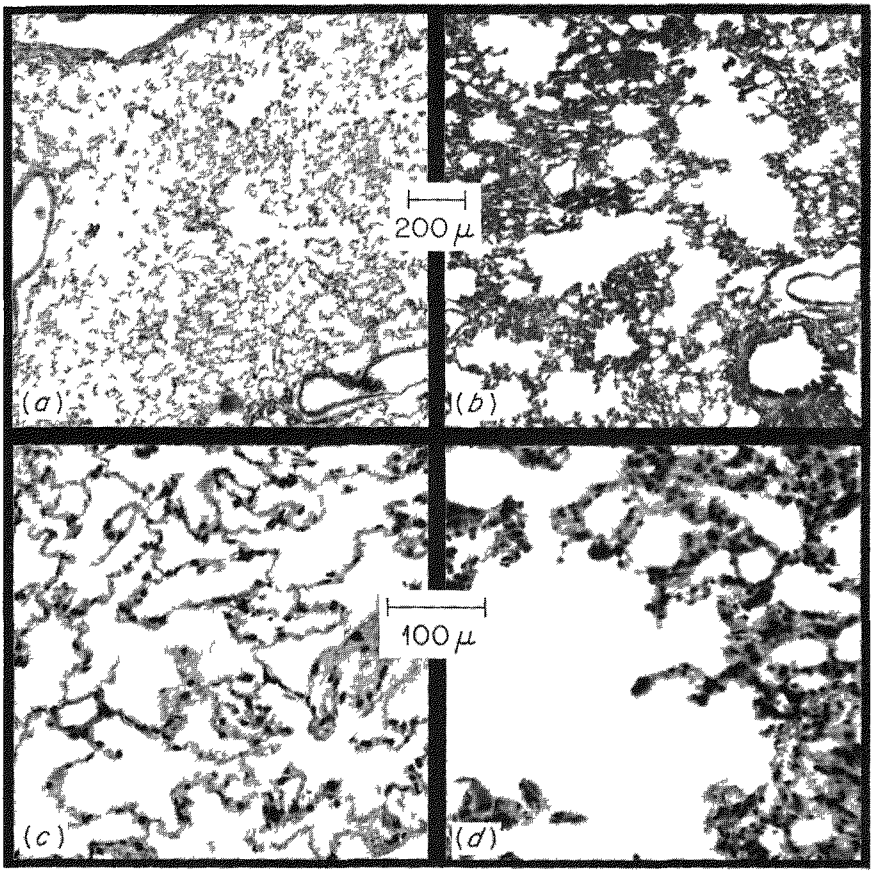

Fig 1 – Comparison of lung parenchyma from control dog 38 (a, c) and experimental dog 26 (b d) Dog 26 had smoked 4084 cigarettes in 423 days

In dog 29 (death on day 24), padlike attachments to alveolar septa were found in all lobes of the lungs Dilatation of the alveolar spaces were present in focal areas within the lungs These were located chiefly in the subpleural aspect of the lungs.

After 229 days of smoking, the changes in the lung of dog 30, although focal, were more advanced The subpleural regions were the predominant sites of change, here the alveolar septa showed a fibrous thickening of their walls, with areas of rupture.

The lungs of dog 35, who died after 278 days of smoking, showed focal subpleural pulmonary fibrosis and ruptured alveolar septa, but to a lesser degree than the previous dog. This was the dog who had to be taken off smoking occasionally and who appeared to inhale the smoke less deeply than other dogs.

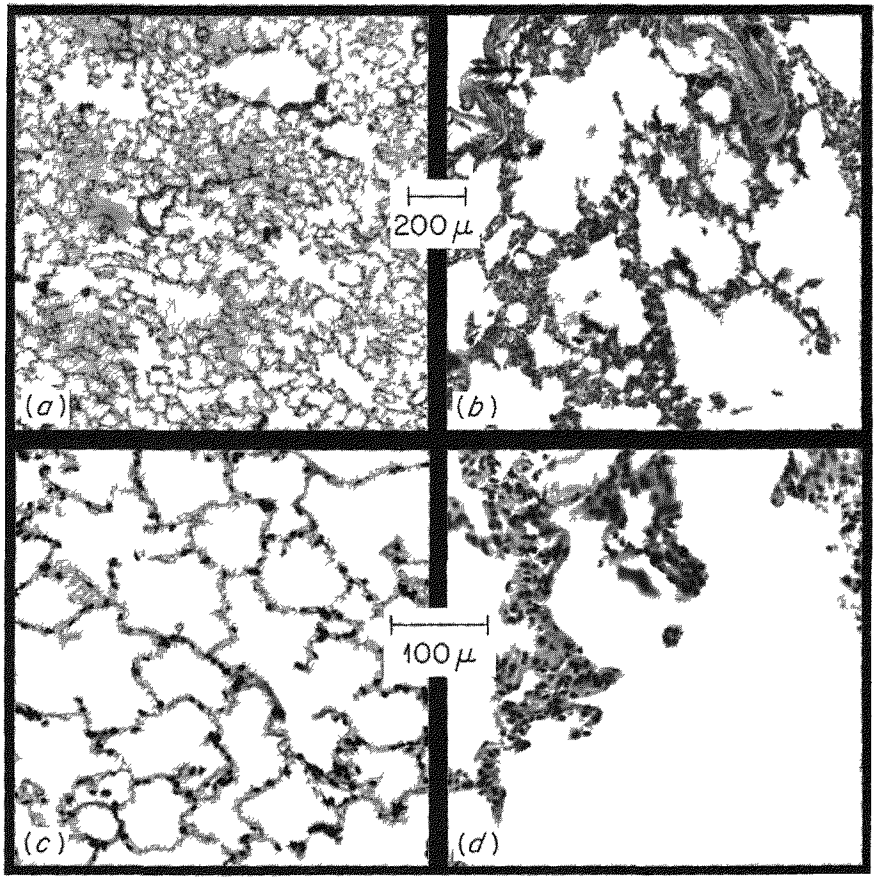

Fig 2 Comparison of lung parenchyma from control dog 42 (a c) and experimental dog 34 (b d) Dog 34 had smoked 4116 cigarettes in 422 days

Dog 32, who died after 410 days of smoking, and dog 36, who died after 415 days of smoking, showed such extensive infarction of the lung parenchyma that it was not possible to evaluate the extent of pulmonary fibrosis and tearing of the alveolar septa in the massive necrotic tissue

Gross and microscopic examination produced fairly consistent findings in the lungs of the remaining five dogs, which were sacrificed after 421, 422 or 423 days of smoking Upon gross examination, the surface of the lungs showed white areas that varied in size from 5 mm to 3 cm These were present over all the lobes but were generally larger in the apical lobes The remainder of the surface had a pink appearance Cross-section of the lung parenchyma revealed the presence of dilated air spaces beneath the pleural surface These measured from 1 to 1 5 mm and gave the lung in these areas a spongy appearance

Microscopically, the white areas showed irregularly arranged zones of connective tissue surrounding dilated air sacs of varying size; within many of these, remnants of alveolar septa were still present (Figs. 1b, d and 2b, d). In other regions of the lung parenchyma, fibrous thickening of the alveolar septa and dilated air sacs was present but was not advanced. There was no thickening of the walls of small arteries and arterioles within the lung.

Figures 1b, d and 2b, d are photomicrographs of the lung parenchyma of dogs 34 and 26. These were selected for presentation as being typical of the changes found in the five experimental dogs sacrificed at the end of the experiment. Photomicrographs of the lung parenchyma of two control dogs are shown for comparison (Figs. 1a, c and 2a, c). Control dog 38 had a tracheostomy and control dog 42 did not.

The lung parenchyma of a number of the experimental dogs showed granulomata of varying sizes. Some of these contained fat, others contained brown pigment, and still others contained both fat and brown pigment. In one of these dogs brown pigment was also present in the regional lymph nodes.

No granulomata were found in the lungs of the control dogs.

HEART WEIGHT

The mean heart weight, expressed in grams per kilogram of body weight, was 7 87 for the 10 control dogs, the individual readings being 6.61, 6.98, 7 09, 7.23, 7.34, 8.08, 8.61, 8.83, 8.96 and 8.99. This is consistent with atypical values for normal male beagles as reported by Hadidian and Pawlowski.[4] The mean for the 10 experimental dogs was 11.94; the individual readings, in the order given for the dogs in Table 1, were 13.22, 15.95, 12.66, 11.37, 8.88, 13.62, 12.01, 9.51, 9.47 and 12.69, respectively. The difference of 4.07 in mean values between the 10 normal dogs and the 10 smoking dogs is statistically significant ($p < 0.001$).

DISCUSSION

Hematocrit studies were made on the dogs because, in a previous experiment on human beings, cigarette smoking was found to be associated with a marked increase in hematocrit; the authors then suggested that this might possibly be related to "the occurrence of diseases characterized by thrombus formation."[6] Hematocrit readings of the dogs increased with smoking (though later they declined somewhat), and several of the dogs died of thrombosis with pulmonary emboli and infarction. Whether these two events were causally related is a matter of conjecture.

Prospective epidemiological studies have indicated a high degree of association between cigarette smoking and death rates from pulmonary emphysema.[7,8] In a histologic study of the lung parenchyma of 1340 men who died of various causes, we found a high degree of association between cigarette smoking and

rupturing of alveolar septa together with fibrosis.[5] Similar findings have been reported in a study of macrosections of human lungs.[9]

Holland *et al.*[10] have reported the occurrence of emphysema in rabbits exposed to cigarette smoke in an exposure chamber daily for from 2 to 5½ years. Hernandez *et al.*[11] exposed greyhound dogs to cigarette smoke in an exposure chamber five times a week for periods ranging up to over 1 year and reported marked changes in lung parenchyma; the changes were related to the duration of exposure.

Instead of using an exposure chamber, we tried to simulate human smoking by having the dogs draw smoke directly from cigarettes into their lungs during two sessions per day 7 days per week.[2] Daily consumption was gradually increased up to the number of cigarettes smoked per day by some extremely heavy human cigarette smokers, body weight being taken into consideration. Advanced changes were found in the lung parenchyma of every one of the five dogs who survived exposure for over 420 days and were then sacrificed. No such changes were found in the lung parenchyma of the 10 unexposed dogs.

The lung parenchymal changes in the experimental dogs showed the characteristics of emphysema found in heavy human cigarette smokers, i.e., rupturing of alveolar septa, fibrous thickening of alveolar septa, and pad-like attachments to the alveolar septa.

In human beings who died at an old age after having been heavy cigarette smokers for many years, we found marked thickening of the walls of arterioles and small arteries in the lung parenchyma.[6] This particular type of change was not found in the lung parenchyma of the smoking dogs, perhaps because the age at which the dogs were sacrificed was less (relative to the normal life expectancy of the species) than the age of death of the human cigarette smokers in whom these changes were observed.

Heart weight per kilogram of body weight was found to be greater in the smoking dogs than in the control dogs. Perhaps this effect was secondary to lung parenchymal changes, as in *cor pulmonale.*

ACKNOWLEDGEMENTS

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REFERENCES

1. O. Auerbach, E. C. Hammond, D. Kirman, and L. Garfinkel, Histological changes in bronchial tubes of cigarette smoking dogs. *Cancer,* 20 (1967) 2055–2066.
2. W. G. Cahan and D. Kirman, An effective system and procedure for cigarette smoking by dogs. *J. Surg. Res.,* 8 (1968) 567–575.
3. M. J. Deubler and E. J. Cole, The microhematocrit in the routine hematologic examination of dogs. *North Am. Vet.,* 38 (1957) 367–377.

4. Z. Hadidian and A. A. Pawlowski, Hematologic, biochemical and organ weight characteristics of beagles. *Cancer Chemotherapy Rep.* 38 (1964) 17–23.
5. O. Auerbach, A. P. Stout, E. C. Hammond, and L. Garfinkel, Smoking habits and age in relation to pulmonary changes. *New Engl. J. Med.*, 269 (1963) 1045–1054.
6. M. E. Eisen and E. C. Hammond, The effect of smoking on packed cell volume, red blood counts, haemoglobin and platelet counts. *Can. Med. Assoc. J.*, 75 (1956) 520–523.
7. E. C. Hammond, Smoking in relation to the death rates of one million men and women. *Natl. Cancer Inst. Monograph,* No. 19 (1966) 127–204.
8. H. A. Kahn, The Dorn study of smoking and mortality among U.S. veterans: Report on eight and one-half years observation, *Natl. Cancer Inst. Monograph,* No. 19 (1966) 1–125.
9. A. E. Anderson, Jr., J. A. Hernandez, P. Eckert, and A. G. Foraker, Emphysema in lung macrosections correlated with smoking habits. *Science,* 144 (1966) 1025–1026.
10. R. N. Holland, E. J. Kozlowski, and L. Booker, The effect of cigarette smoke on the respiratory system of the rabbit: A final report. *Cancer,* 16 (1963) 612–615.
11. J. A. Hernandez, A. E. Anderson, Jr., W. L. Holmes, and A. G. Foraker, Pulmonary parenchymal defects in dogs following prolonged cigarette smoke exposure. *Am. Rev. Respirat. Diseases,* 93 (1966) 78–83.

DISCUSSION

G. Poda: A matter of practicality, have you had any dogs that you allowed to smoke and then, after a period of no smoking, examined them to find out whether the people who have quit smoking cigarettes can quit worrying, or something like that?

O. Auerbach: In the new study that we are now doing, we had a number of things that we were trying to do. Number one was to look at the effects of light smoking versus heavy smoking, as well as unfiltered smoking and heavy, filtered smoking versus heavy, unfiltered smoking. One of the other things we were going to do was to see how far we could let the dogs smoke, and then see what had developed within their lungs. Unfortunately, we reached the point of no return; we could not let them continue to smoke, so we began to sacrifice. But we've done a study on human beings and have found, by comparing 72 cigarette smokers with 72 exsmokers and with 72 nonsmokers, that there was a 40-fold drop in cells with atypical nuclei and precancerous lesions in persons who had stopped smoking for at least 5 years. This aspect was also part of our design in the study on dogs, but it could not be fulfilled.

D. L. Coffin: This was my first opportunity to see your work presented first-hand, and I think it is a beautiful study. I couldn't help but be reminded of the somewhat comparable effects produced in our own laboratory by exposure of dogs to nitrogen dioxide at 26 ppm for 6 months; in Dr. Gus Freeman's laboratory by exposure of rats and monkeys at 12–25 ppm NO_2; and in Richard Ehrlich's laboratory by chronic exposure of mice at 0.5 ppm NO_2. It therefore seems to me that there may be some common denominator between the effects that are produced with tobacco smoke in your laboratory and in the other

laboratories with NO_2 exposure. I was therefore wondering if you could tell us the NO_2 content of your smoke.

Auerbach: Fitelson Laboratories have made an analysis of the cigarettes used in our dog study, and the results are being kept from me until the study is over. In this new study, a half-million cigarettes were bought and were examined by Fitelson. The analysis will appear in our published results of the current study. We are hoping to be able to compare the effects produced by exposure to nitrogen dioxide and cigarette smoke in this third study that we are undertaking.

F. J. C. Roe: Did your control dogs have tracheostomies?

Auerbach: Two of the control animals in the preliminary study did, and all of the controls in the second study have them. We tried sham smoking with these animals, but the turnover in personnel was so great that we could not continue.

Roe: What is the significance of the pulmonary infarction, which is not regarded as a consequence of smoking in man?

Auerbach: Well, the big question is: How much of a role does the smoking play in thrombosis? Unfortunately, we don't have the physiologists or hematologists to work with us on this. We would love to have the answer. Even in this new study where we have been allowing the dogs to smoke up to nine cigarettes a day over a longer period of time, we still get infarction, but much less. We don't have the answer.

K. H. Kilburn: Your study is particularly provocative to those of us who are emphysema-oriented. I wonder if you would tell us what the definition of emphysema is.

Auerbach: I don't know whether I'm being mouse-trapped, but I'll tell you a little story about that! I was on a program at the A.M.A., and I showed slides just like this. One of the men whom you know very well, and who will remain anonymous, was on the program with me, and he was asked to give his definition of emphysema. He said, "Rupture and tearing of the alveolar septum." Then he turned to me, and I said, "Rupture and tearing of the walls of the alveolar septum, that's emphysema." Then the chairman said, "You both agree." That was in the first part of the program. Later on I showed these same slides, and the moderator turned to this gentleman, and asked, "Is this emphysema?" And he said, "No, it's not."

But let me say that I've shown these slides to many of my colleagues in the field of pathology – my good friends Dr. Marvin Kuschner, Dr. Averill Liebow, and a number of others – and they haven't quarreled with me about whether this was or was not emphysema. So if you're willing to accept that definition, we have produced it.

H. G. Boren: I think it would be a shame, in the context of this meeting, if Dr. Auerbach didn't say something about changes in the bronchial epithelium, which I think are really more pertinent to this group. I may be out of order, Dr. Nelson, but I think he should summarize his observations on these.

N. Nelson: I think that's an appropriate nudge!

Auerbach: In the preliminary study we produced a series of changes in the bronchial epithelium that eventually led to keratosis. In other words, we produced all of the atypical changes. In our present study we have sacrificed 40 animals; 32 are smokers and 8 are not. I came to this meeting 2 days late because I have been describing the findings in these sacrificed animals. We are cutting 166 slides from each tracheobronchial tree. The slides will be on my desk tonight. I suppose it's a little premature, but I can tell you that at this point I have nothing to retract from the first study.

In the dogs that died earlier in the course of this study, we've found changes similar to the findings in the dogs in the first study. We hope to do much better with the sacrificed animals.

R. Rylander: I just wanted to ask you one question. We found that a great proportion of the water-soluble substances in the exposure agent, the smoke itself, do get absorbed in the mouth. It's something I've been saying for a couple of days now! Have you intended to do any experiments where you would pass the smoke through some sort of moistened tube, or another device that simulates the oral cavity?

Auerbach: I wish we could take all of the advice and suggestions people give us – and I don't mean that facetiously – but anybody who attempts this kind of study with the help that is available today should have his head examined! I really mean it! You know I've been up against labor unions and what have you, and with the turnover of help, we are fortunate even to have a study. Your suggestion is well taken, and if we are able to do it, we certainly will try.

D. Craig: Mr. Chairman, the comment that I want to make really concerns everyone who has shown histological slides, but in particular I was greatly bothered by the comparative slides that Dr. Auerbach showed of dogs that had smoked and those that had not. It seems to me that there was a difference in the quality of the staining, or the eventual color of the staining. I want to know, is it not possible to put some sort of internal standard into histological slides, so that one can distinguish exactly what the quality of the staining is?

Auerbach: Your point is well taken, and the quality is important. These are thin sections, and sometimes the eosin gets a little bit heavier on some than on others. I will see if we can't overcome that handicap, too.

EXPERIMENTAL INVESTIGATIONS ON THE EFFECT OF CIGARETTE SMOKE INHALATION ON SMALL LABORATORY ANIMALS

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ABSTRACT

Experiments were performed on hamsters, mice, and rats in an effort to find a practicable inhalation model for long term exposure to tobacco smoke These experiments were designed to reveal all reactions of the animals during passive smoke exposure so that the effects of passive smoking on animals could be compared with the effects of active smoking on man The toxicity of several smoke components, such as CO and nicotine, and the compatibility of different exposure cycles or dosages were investigated, influences on respiratory frequency, counts of erythrocytes and leukocytes, sedimentation rate, body weight, and food intake during smoke exposure were also measured Moreover, we have tried experimentally to clarify the significance of the stay period of carcinogenic substances in the lung as well as the metabolism of carcinogenic substances Histological changes in the respiratory tract following long-term exposure to high smoke doses are reported By measuring assimilated ^{14}C-labeled smoke particles, we have tried to explain the genesis of the alterations that we found

Long-term experiments with Syrian golden hamsters, completed in our laboratories in 1961,[1-3] demonstrated a remarkable organotropy in the respiratory tract following application of nitrosamines Experiments were therefore undertaken to investigate the suitability of these hamsters for testing other identified or suspected carcinogenic substances

We began our inhalation experiments about 10 years ago [4] Syrian hamsters were exposed to cigarette smoke by means of a smoking chamber that was relatively large and of the type described by Essenberg[5] (Fig 1) Soon, however, we realized that this model system for inhalation studies results in a very low concentration of smoke in the chamber, particularly at the beginning of the experiment In addition to the insufficient concentration of potent particulates in the smoking chamber, there are several other factors to be considered, such as the possibility of nasal filtration and the prolonged or altered passage of inhaled smoke through the nasal cavity

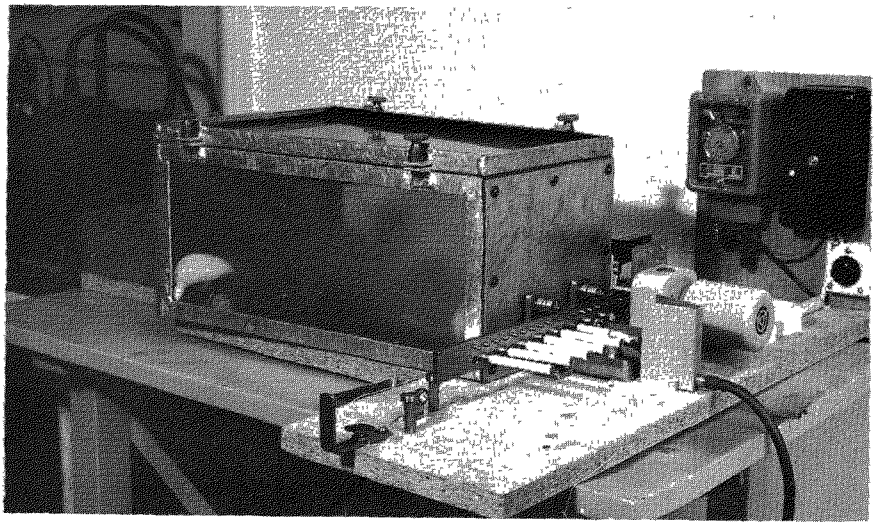


Fig. 1 – Smoking machine with a large vacuum chamber designed for 10 hamsters ("closed smoking system"). (From Essenberg[5])

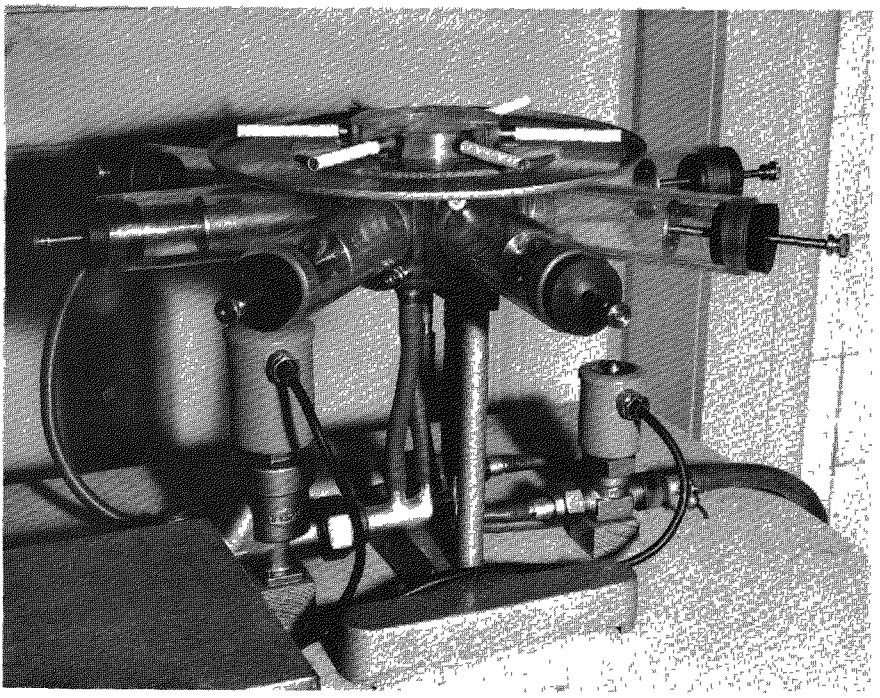


Fig. 2 – Smoking machine with automatic smoke/air ventilation, designed for eight hamsters (type Dontenwill – "closed smoking system"). (From Dontenwill *et al.*[7])

In our opinion there were two problems that had to be solved (1) designing a smoking chamber device that produces and offers the animals high concentrations of smoke without endangering them under experimental conditions, CO poisoning and nicotine intoxication were especially to be prevented, and (2) proving, in experimental studies, the assimilation of smoke in the respiratory organs and the effects of smoke components on the respiratory tract.

During the Third Quadrennial Conference on Cancer, held in Perugia in 1965, we[6] reported results of experiments with a new type of smoking machine that was based on the closed system (type Dontenwill). The exposure unit (Fig. 2) was so small that the hamsters were exposed to a highly concentrated atmosphere of smoke and air. The machine provided a smoking cycle with conditions standardized according to human smoking habits. Results of these experiments revealed some papillary proliferations of the tracheal epithelium as well as metaplastic changes, including atypical nuclei and severe proliferation in the upper part of the trachea, these histopathologic alterations seemed to be comparable with precancerous changes

In 1965 we began experimental studies[7] on the intake and metabolism of CO (Fig. 3) in different smoking systems, which we called the "closed" and the "open" systems; these studies showed that an open smoking system contains little risk for CO intoxication. After thoroughly investigating the formation of CO-hemoglobin and the assimilation of nicotine into the lungs and other organs

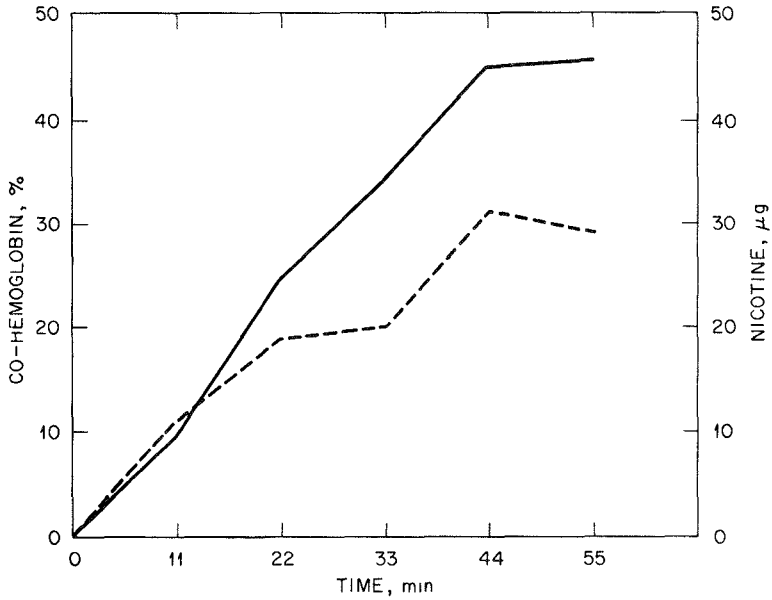


Fig. 3 – Increase of CO-hemoglobin (——) in the blood and nicotine (--) in the lungs of hamsters exposed to smoke in the smoking chamber type Hamburg (From Dontenwill *et al* [8])

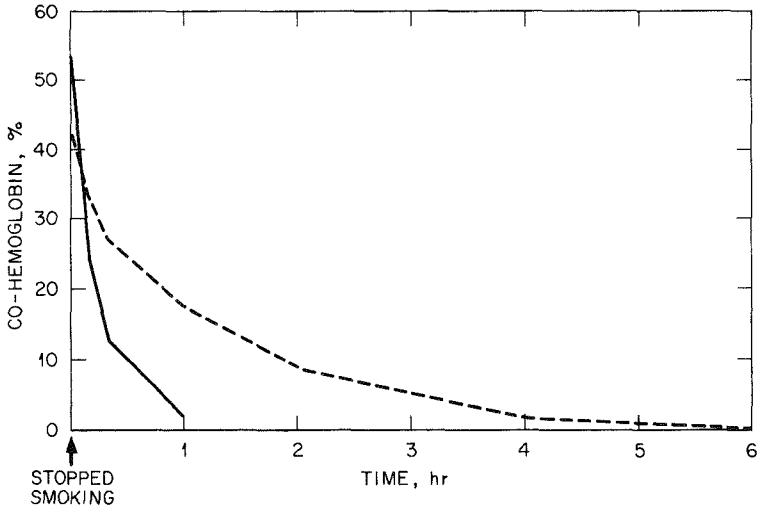


Fig. 4 – Reduction of CO-hemoglobin in the blood of smoke-exposed hamsters: in the presence of fresh air (---) or after addition of O_2 to the offered air volume (——). (From Dontenwill *et al.*[7])

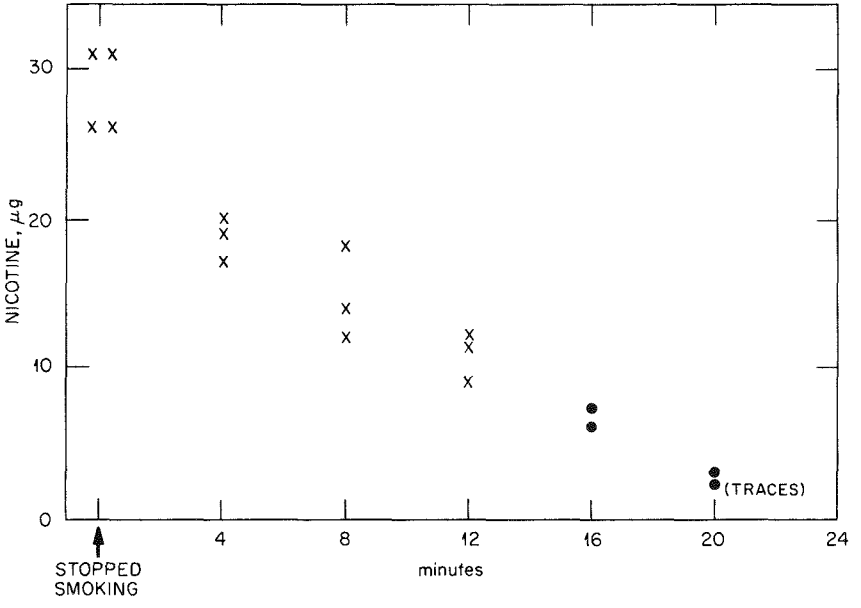


Fig. 5 – Reduction of nicotine in the hamster lung after smoke exposure. x = mean from three animals; • = mean from six animals. (From Dontenwill *et al.*[7])

in different inhalation systems (Figs. 4 and 5), we developed a new smoking machine, type Hamburg. This machine has significant advantages, especially for long-term inhalation studies[8] (Figs. 6 and 7):

1. It is possible to expose animals to cigarette smoke over a long period of time. At present, we have had more than 2 years of experience with this type of machine.

2. The machine offers fresh smoke in concentrations and quality that remain constant during the course of experiment, and it guarantees a uniform rhythm of smoke flow. Moreover, the distance between cigarette and animal is kept as small as possible.

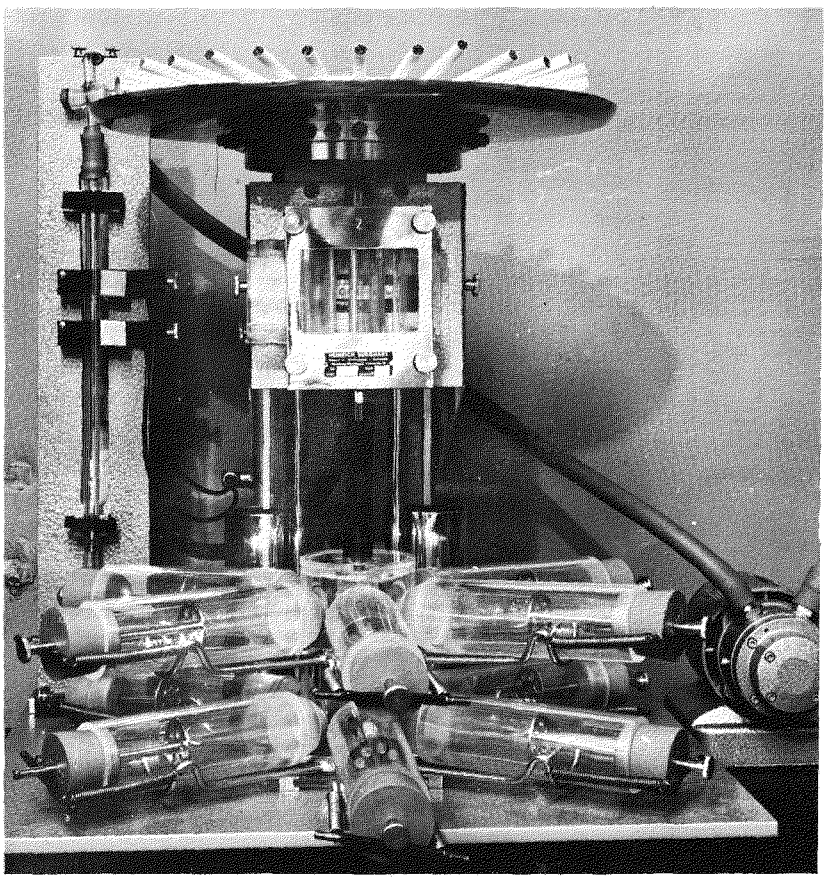


Fig. 6 – Smoking machine type Hamburg ("open smoking system"), adaptable to either 10 hamsters, 10 rats, or 10 mice. The concentration of the smoke/air admixture is adjustable, and the puff volume is automatically controlled. (From Reckzeh *et al.*[9])

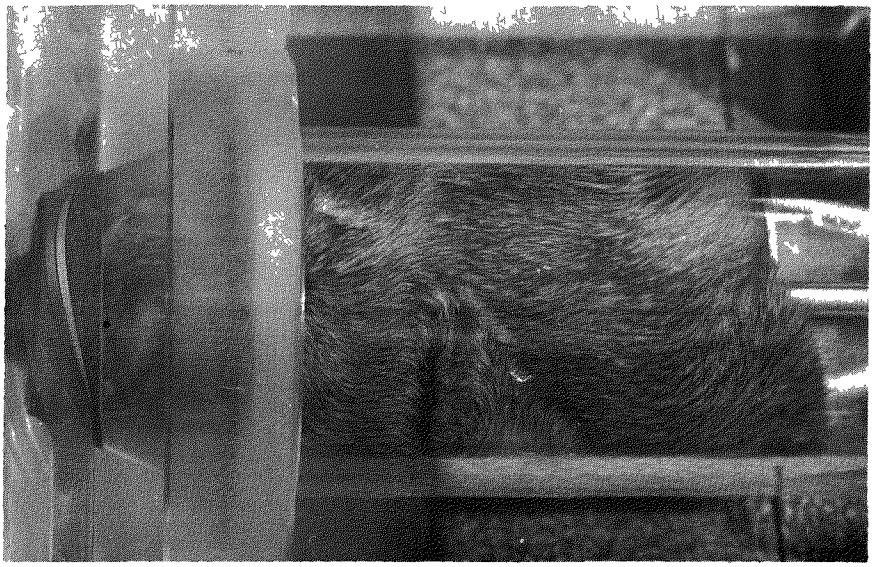


Fig. 7 – Animal-holding tube of the smoking machine type Hamburg. Within the tube are immobilization equipment and an opening into the smoking chamber for the nose and mouth of the hamster.

3. Pilot studies have determined the smoking cycles that assure the most effective dose at lowest toxicity (Table 1). It has been possible to demonstrate experimentally the rising concentration of CO (ref. 9), which is a limiting factor in long-term inhalation tests. Nicotine, on the other hand, is tolerated even in high doses, but the dose tolerated varies according to the species tested.

TABLE 1

*Smoking Cycle, Duration of Exposure to Smoke, Number of Cigarettes Smoked, and LD_{50} of Hamsters Smoking Reference Cigarettes in the Smoking Machine Type Hamburg**

Smoking cycle (cigarettes/day)	LD_{50} time (weeks)	Days† of exposure to smoke/ number of cigarettes smoked
3 X 60	1.5	7/1350
3 X 30	34.0	170/15,300
1 X 60	39.0	195/11,700
1 X 90	30.0	150/13,500
2 X 30	>34.0	>170/10,200‡
Untreated controls	>52.0	

*From Reckzeh *et al.*[9]
†Five days a week.
‡Experiments in progress.

4. According to inhalation experiments carried out with aerosols and to investigations on the intake of nicotine when the nose is opened or closed, it was demonstrated that one could count on high smoke assimilation by selecting an inhalation apparatus that is technically appropriate.

When experimental cigarette smoke inhalation in animals is to be compared with active smoking in man, many factors must be carefully controlled and considered to assure that cigarettes "smoked" by animals copy actual human smoking patterns as closely as possible.

In studying the respiratory frequency of hamsters in the "closed" and in the "open" smoking systems, we[10] found that respiratory frequency as well as respiratory volume is increasingly diminished during long periods of inhalation and that this effect is especially pronounced in "closed" smoking systems (Fig. 8). Accordingly, the assimilation of smoke decreases. When animals are exposed to cigarette smoke in "open" smoking systems (Fig. 9), respiratory frequency is not affected to so great an extent. According to our experience there are two limiting factors for inhalation experiments: CO-hemoglobin concentration and respiratory function of the test animals.

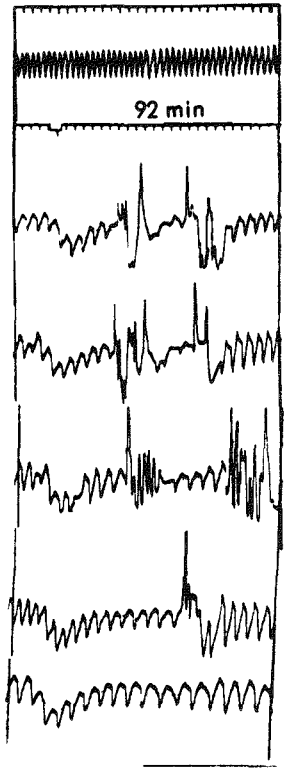


Fig. 8 – Respiratory frequency of hamsters after smoke exposure in a "closed system" (type Dontenwill). Reflectory stop of respiration is seen after beginning of smoke exposure. (From Rucker and Dontenwill[10])

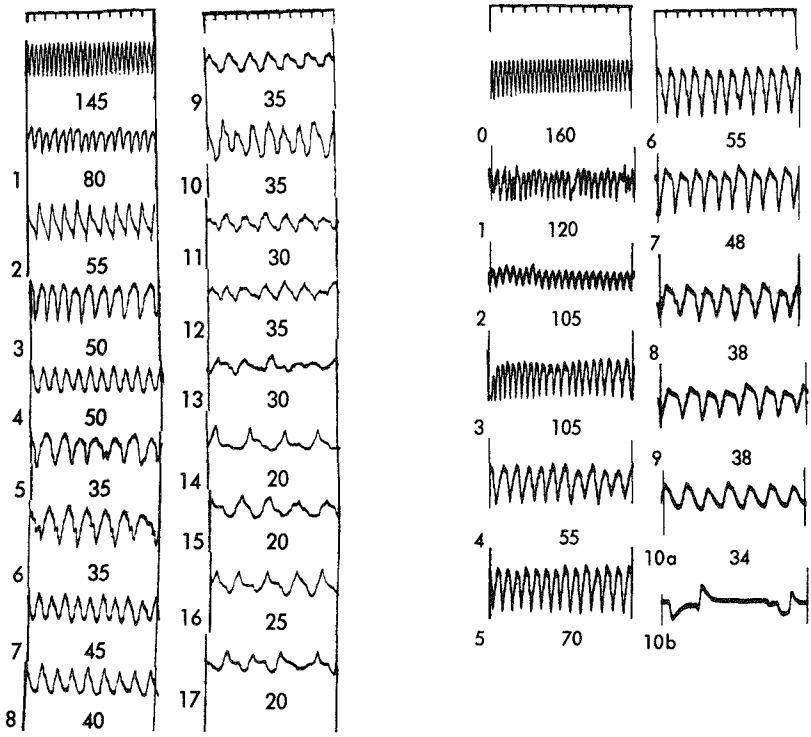


Fig. 9 – Respiratory frequency of two hamsters after smoke exposure in the "open smoking system." Reduction of respiratory frequency after continuous smoke exposure can be seen. Duration of smoking, 170 or 100 min. Numbers inside graph indicate respiratory frequency per minute; those on the outside indicate number of plates (30 cigarettes each). (From Rücker and Dontenwill[10])

When responses of experimental animals to CO are to be interpreted for comparison with human responses, it must be stressed that CO-Hb values occurring relatively quickly in animals during passive smoking are never reached in man after active smoking. As we have recently reported,[11] the number of erythrocytes is significantly increased after hamsters are exposed to cigarette smoke; this increase probably can be explained by a reduction of the oxygen-transporting hemoglobin. However, no relationship between smoking and alteration in white cell count could be demonstrated (Fig. 10). The sedimentation rate in hamsters is not accelerated during exposure to cigarette smoke. Investigations of body weight development[9-11] distinctly indicated that animals exposed to cigarette smoke reached, and maintained throughout the experimental period, a significantly lower body weight that was associated with decreased food consumption. In fact, detailed inspection of growth curves indicates that days without smoking are associated with a slightly increased body weight (Fig. 11). These experiments again show the appetite-inhibiting effect of smoke.

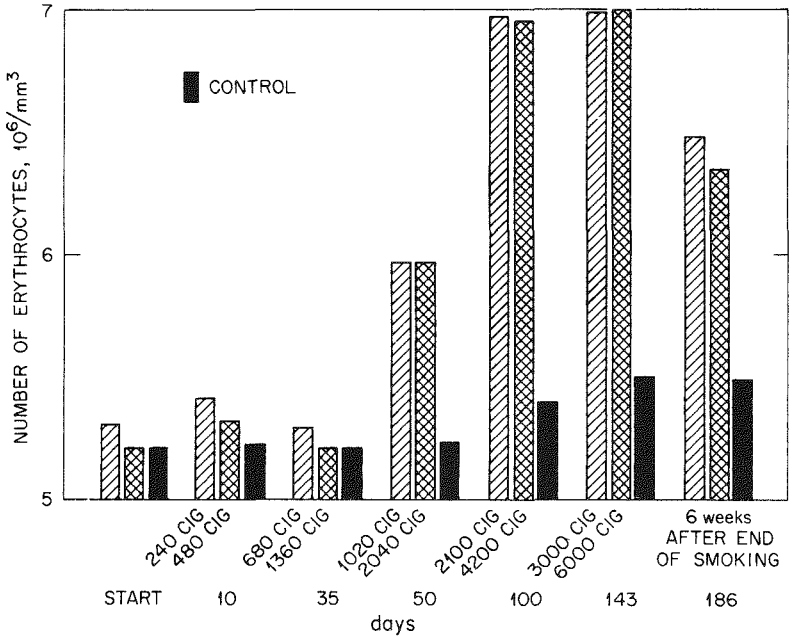


Fig. 10 – Effect of cigarette smoke on erythrocyte count in hamsters (From Reckzeh and Dontenwill[11])

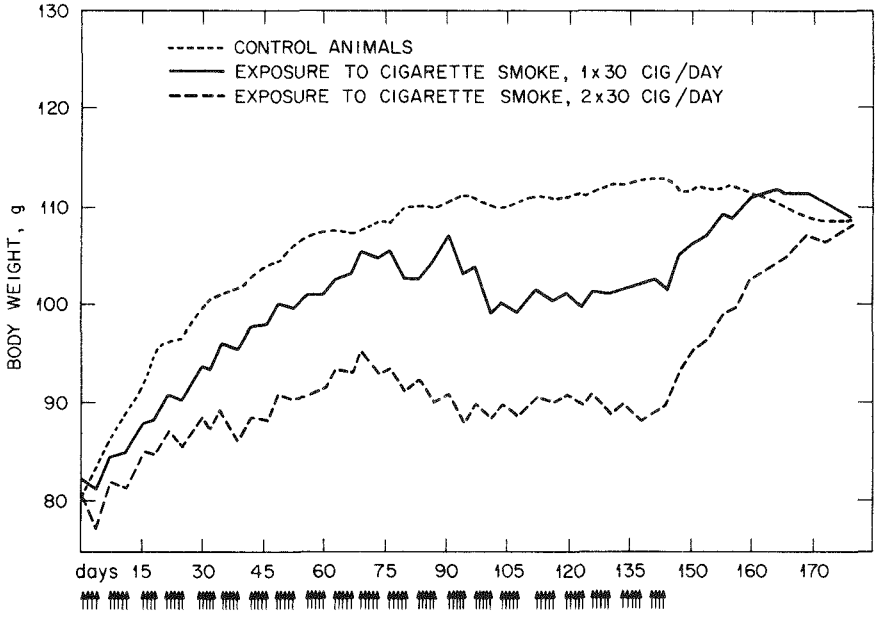


Fig 11 – Effect of cigarette smoke on the hamster's development of body weight Arrows indicate number of exposures (From Reckzeh *et al* [9])

TABLE 2

*Studies of Adaptive Zymogenesis in Smoke-Exposed Rats**

Dose of zoxazolamine† (mg/kg)	Pretreatment	Time of paralysis‡ (min)	Controls‡	Difference (%)
100	12 Exposures	146 (4/4)	177 (4/4)	18
160	to smoke of	234 (19/20)	335 (18/20)	30
160	3 X 30 cigarettes daily	232 (18/26)	388 (27/30)	40
100	Injection of 40 mg/kg cigarette smoke condensate i.p.	79 (13/15)	140 (11/15)	44
100	10 mg/kg	44 (15/30)	143 (20/30)	69
100	benzpyrene i.p.	23 (6/10)	87 (8/10)	74
160	Partial resection	262 (9/9)	192 (8/9)	36
160	of the liver	700 (8/9)	298 (9/9) §	135

*From Dontenwill *et al.*[12]

†2-amino-5-chlorobenzoxazole.

‡Numbers in parentheses indicate animals with paralysis per total experimental animals.

§Sham operation – opening of the peritoneal cavity.

TABLE 3

*Studies of Adaptive Zymogenesis in Smoke-Exposed Hamsters**

Dose of zoxazolamine† (mg/kg)	Pretreatment	Time of paralysis‡ (min)	Controls‡	Difference (%)
100	12 Exposures	49 (16/20)	67 (18/20)	27
160	to smoke of 3 X 30 cigarettes daily	128 (15/20)	163 (18/20)	22
100	Injection of 40 mg/kg cigarette smoke condensate i.p.	48 (15/20)	57 (13/20)	16
100	10 mg/kg	49 (14/20)	57 (13/20)	14
125	benzpyrene i.p.	51 (8/10)	73 (9/10)	30
125		51 (29/30)	61 (28/30)	16

*From Dontenwill *et al.*[12]

†2-amino-5-chlorobenzoxazole.

‡Numbers in parentheses indicate animals with paralysis per total experimental animals.

Studies of adaptive zymogenesis following smoke exposure[12] proved that the formation of hydroxylase is intensified after smoke exposure (Tables 2 and 3), and that the hydroxylase is able to eliminate aromatic hydrocarbons. Our observations have already been confirmed by Welch *et al.*[13] after investigations in man. As Kotin *et al.*[14] have pointed out, this process of elimination, which obviously functions very well in hamsters, may be decisive for the effect of carcinogenic substances.

In order to keep toxicity as low as possible during inhalation experiments, several test cigarettes were used on different animal species to determine the acute and chronic toxicity of certain smoke components.[9] The results we obtained have a primary bearing only on the animal experiment because, as previously stated, a CO-response occurring during passive smoking in animals is never reached during active smoking in man. Nevertheless, we trust that some results from the experiment may be applied to man. These toxicity studies demonstrate the following (Tables 4 and 5):

TABLE 4

*Comparative Studies on Toxicity of Reference and Modified Cigarettes (Smoking Machine Type Hamburg), with Particular Regard to Survival Time and CO and Nicotine Contents of the Cigarettes**

Type of cigarette	Vol. CO (%)	Nicotine content of smoke (mg/cig.)	Number of puffs per plate†	Time of exposure to smoke until death (min)			
				Hamster	Rat	Mouse (ICI)	Mouse (BALB/c Jax)
E (standard)	4.6	2.65	10	184	16	76	15
ET (humidity $< 6\%$)	4.3	2.60	10	117	12	33	16
E_x (extracted with 70% ethanol)	5.1	0.68	10	176	36	64	38
EG_1 (E – reconstituted tobacco)	7.7	0.82	8	25	8	8	12
EG_3 (EG_1 + $NaNO_3$)	7.0	0.47	7	21	8	6	14
E_5 (E – tobacco stems)	5.3	0.36	9	71	24	43	39
E_6 (reconstituted tobacco from E_5)	11.1	0.64	10	26	11	11	13
K (E + charcoal filter)	5.1	1.68	10	114	17	25	19
A (E + acetate filter)	4.2	1.31	10	160	27	30	40
E + Cambridge filter	4.6	0	10	147	158		
$E_6 + O_2$		0.64	10	49	22	37	
$ET + O_2$		2.60	10		20		

*From Reckzeh *et al.*[9]

†One plate holds 30 cigarettes.

TABLE 5

*Comparative Studies on Toxicity of Cigarillos**

Type of cigarillo	Vol. CO (%)	Nicotine content of smoke (mg/cig.)	Number of puffs per plate	Time of exposure to smoke until death (min)			
				Hamster	Rat	Mouse (ICI)	Mouse (BALB/c Jax)
Cigarillo I	12.3	2.9	20	53	9	10	
Cigarillo I + O_2		2.9	20	100	26	12	
Cigarillo I + Cambridge filter	12.3	0	20	63	15	19	
Cigarillo II	12.0	2.5	20	67	24	21	16

*See Fig. 14.

1. Comparative investigations of the gaseous and the particulate phases indicate that the CO response is equal in animals such as hamsters, mice, and rats.

2. Hamsters are not at all as susceptible to nicotine as are rats and certain strains of mice.

3. Test cigarettes with a very high CO level are unsuitable for chronic inhalation experiments.

4. There seems to be a summation effect of CO and nicotine. These findings, as well as all the other results mentioned, enabled us to determine an optimal dosage for our inhalation experiments. In this connection, we would like to point out that the decomposition of nicotine, for example, in the hamster liver, is significantly accelerated as compared with the decomposition of nicotine in the rat liver *in vitro.*[15]

Actually, the hamster was found to be the most suitable test animal for inhalation experiments, in part because of its resistance to pulmonary infections and its ability to decompose nicotine, and especially because of the type and location of the experimentally induced lung tumors. In this connection, investigations of Saffiotti *et al.*,[16] Herrold and Dunham,[17,18] and Kuschner[19] may be cited.

Before the effect of long-term exposure on the respiratory tract of hamsters is reported, a few examples should be mentioned to demonstrate the primary design of experiments. We are very grateful to the monograph of Wynder and Hoffmann,[20] which places considerable emphasis on the development of a "less harmful cigarette" or "a safer cigarette" and collects so many contributions of authoritative discussions for these special areas. Our own endeavors, too, have been primarily aimed at determining possible risks of tobacco smoking, as well as finding a way of eliminating or reducing harmful substances. When observations of a reduced tumorigenic activity of sodium nitrate and of certain tobacco sheets were published,[20] it was strictly advised that this additive should not be used

because a new risk would be possible if it were. Druckrey and Preussmann[21] and others have repeatedly pointed out that the addition of sodium nitrate may be the cause of an increased formation and uptake of nitrosamines. We cannot yet answer the question of whether the addition of sodium nitrate to tobacco possibly inhibits the tumorigenic activity only in the area of the skin, or whether the activity of this additive is enhanced in the lungs. These considerations provided a valuable impetus toward designing an important model system for animal inhalation studies on total smoke.

In our total program of experimental inhalation studies, the principal stress was given to the assumption that tobacco smoke must lead to a definite reaction at some place on the respiratory tract when administered in sufficiently high quantities to the animals. The testing of this working hypothesis seemed to us urgently necessary. In the case that strong nasal filtration leads to a considerable qualitative and quantitative reduction of the smoke assimilated, the reaction must appear in the area of nasal passages; or, in the case that sufficient particles have passed the nasal region, the reaction must appear at a more distant place in the respiratory tract, i.e., at a place where physically the highest concentration is to be expected.

In terms of the so-called smoke-passage leading from the nostril to the lung periphery, we have histologically investigated serial sections of this portion of the respiratory tract. Histological observations in the trachea, bronchi, and alveoli reconfirmed results which had already been proven by us and other investigators. The nasal cavity (Fig. 12) and the trachea exhibited inflammatory mucosal changes as well as epithelial metaplasia, especially in animals which had been exposed to smoke for a long time. Small typical papillomas were found in the nasal cavity of two animals exposed to smoke for a long time. Alveoli (Fig. 13) showed a concentration of macrophages with brownish epithelial inclusions, which were defined by Otto[22] as "smoke-cells." A few animals that were exposed to smoke for a long time showed small "adenomatoid lesions" in the lung.[16] Critical examination of findings in the nasal cavity, trachea, and lung is unwarranted in the context of the total results. Therefore, we shall not discuss these findings in detail but will turn, instead, to the larynx, which had, as far as we have seen, the most remarkable alterations.

For chronic smoke exposure, 146 hamsters were selected. As shown in Fig. 14, 93 animals survived smoke exposure for more than 10 months. The figure allows not only an interpretation of survival chances in the course of long-term exposure but also a temporal interpretation of ascertained histological changes.

In order to have better control of the induced changes, we differentiated five stages of epithelial changes in the larynx (Table 6), using the *Atlas of Tumor Pathology* of the Armed Forces Institute of Pathology[23] as reference.

As in human pathology, a differentiation of so-called pseudoepitheliomatous changes and the onset of carcinomas in the larynx is very difficult. Anyone with long experience in this field and with the above-mentioned atlas or other literature on this subject in mind knows that the criterion for malignancy in the

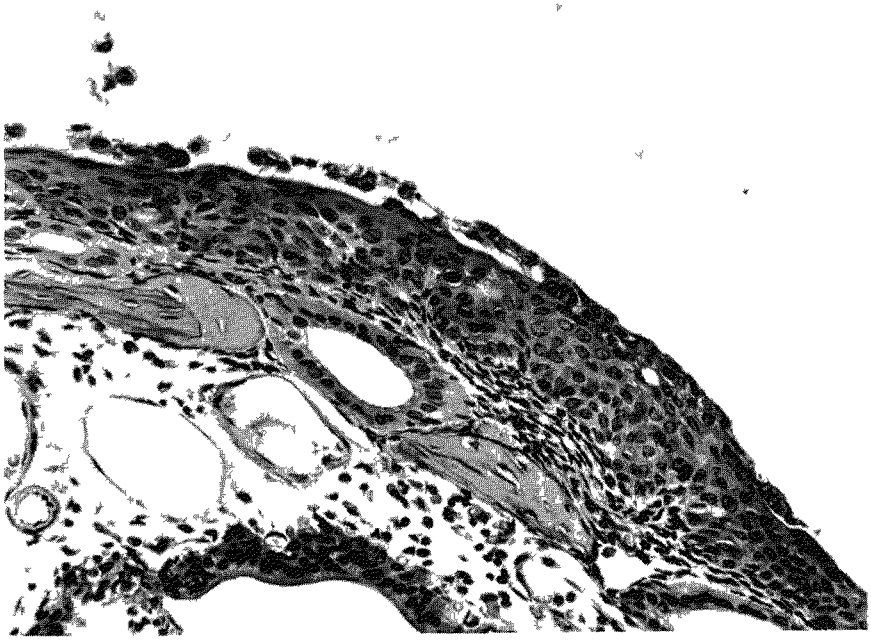


Fig 12 – Squamous cell metaplasia of the nasal cavity mucosa after exposure to smoke for 58 weeks

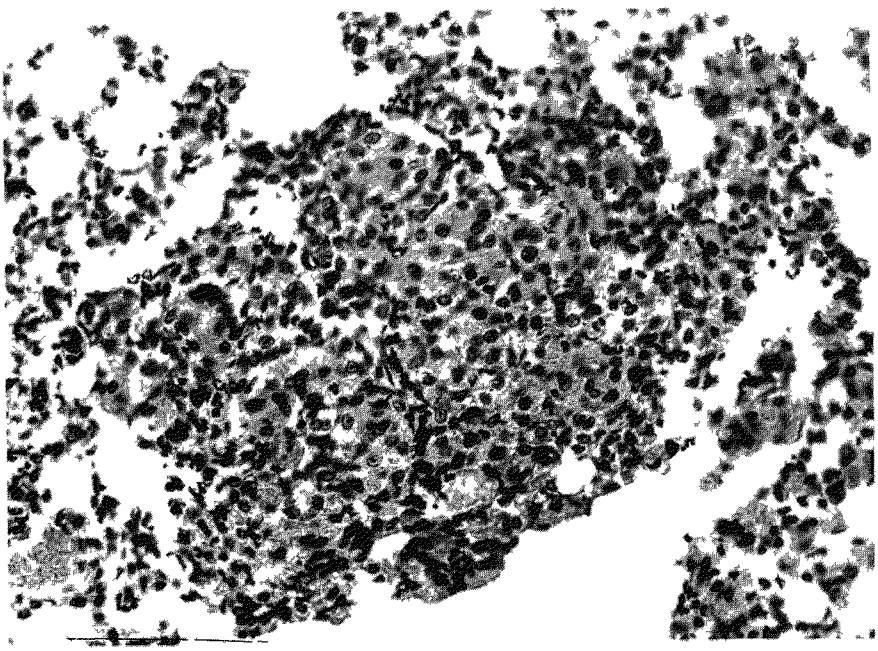


Fig 13 – Accumulation of macrophages in the lung of a smoke-exposed hamster (91 weeks) Brownish epithelial inclusions are prominent

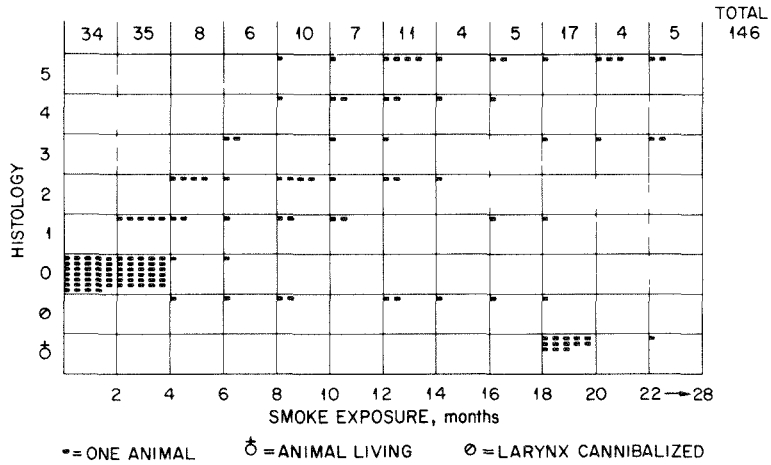


Fig. 14 – Review of the results of our inhalation experiments: number of smoke-exposed animals with and without changes in the larynx (Stages 1–5; see Table 6 for description); duration of smoke exposure and number of animals still alive.

TABLE 6

*Classification of the Five Registered Stages of Epithelial Changes at the Larynx**,†*

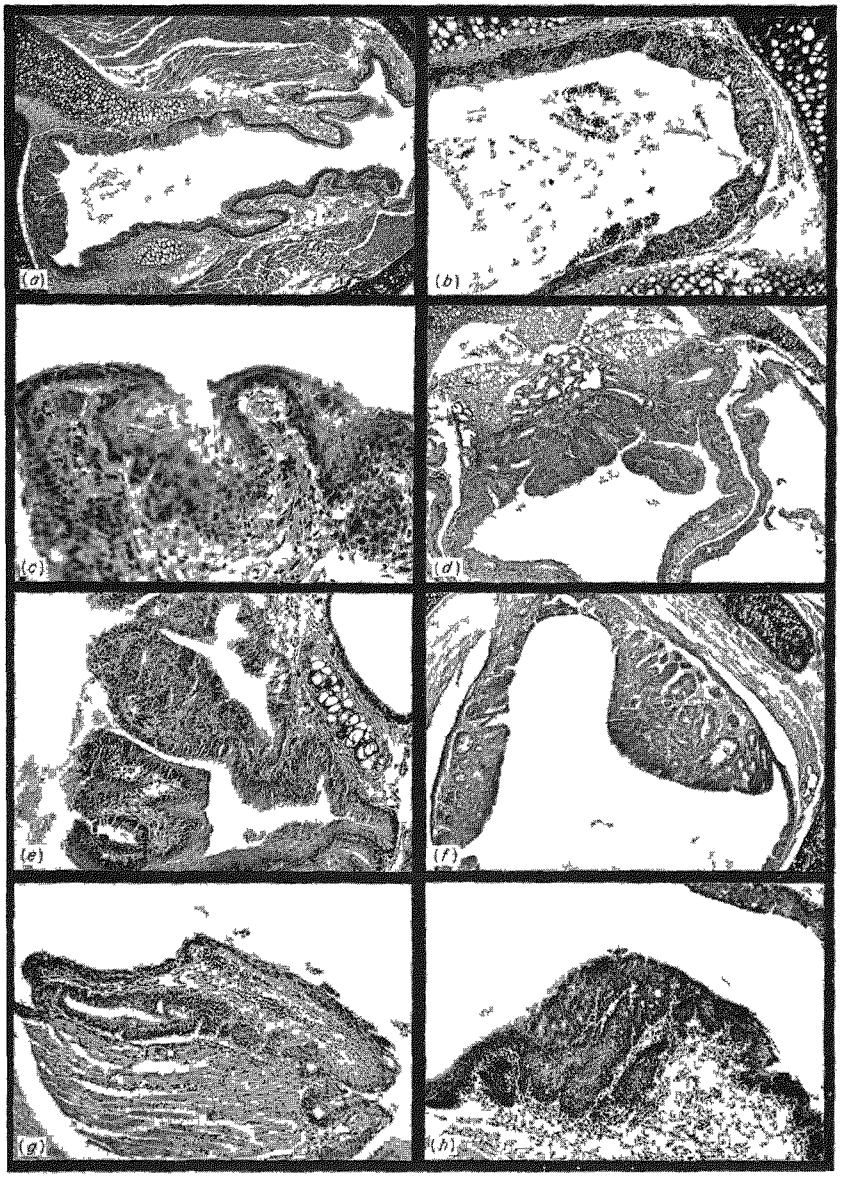
Stage	Acanthosis (thickening of stratum spinosum multicellular layer)	Hyperkeratosis increased cornification (stratum corneum)	Parakeratosis (incomplete cornification of nuclei in the stratum corneum)	Dyskeratosis (premature atypical cornification changes in the nucleus proliferation of the basal layer)	Mitosis
1. Pachydermia (epithelial hyperplasia)	+	+	∅	∅	∅
2. Leucoplakia	+	+	(+)	(+)	(+)
3. Verrucous leucoplakia	+	+	+	(+)	(+)
4. Papillomatous leucoplakia	+	∅	∅	++	(+)
5. Pseudoepitheliomatous leucoplakia	+	+	+	+++	+

*Symbols: ∅ = negative; + = weak; ++ = medium; +++ = strong; (+) = minimal.
†From *Atlas of Tumor Pathology*.[23]

larynx is very difficult to explain. We intentionally avoid the term "carcinoma *in situ*," but would like to point out that several of the five classified findings are obligatory precancerous stages. In order to convey an impression of ascertained epithelial changes, we have shown some examples of very severe stages (Figs. 15a–q). Figure 15r, however, shows an early invasive carcinoma.

[Editors' note: Figure 15r is an addendum to the original presentation and discussion at the conference.]

Fig 15 – Epithelial changes in the larynx See Table 6 for classification of the various stages ET exposure time (a) Pachydermia of the larynx (ET = 24 weeks) (b c) leucoplakia of the larynx (ET = 38 and 56 weeks respectively), (d) verrucuous leucoplakia of the larynx (ET = 73 weeks) (e f) papillomatous leucoplakia of the larynx (ET = 54 and 72 weeks respectively) (g–q) pseudoepitheliomatous leucoplakia (hyperplasia) of the larynx (ET for g = 55 weeks for h 51, for i 65, for j and k 73 for l m, and n 99, for o 55, and for p and q 72 weeks) (r) early invasive carcinoma (ET = 72 weeks)



(i)
(j)
(k)
(l)
(m)
(n)
(o)
(p)
(q)
(r)

Figure 14 clearly shows that (1) only a few animals are surviving after a sufficiently long period of exposure; (2) all animals exposed to smoke for more than 10 months exhibited changes in the area of the larynx; (3) the degree of severity in the changes corresponds to the duration, concentration, and frequency of exposure; and (4) the smoking cycle is, therefore, of utmost importance in the outcome of the experiment.

What may be concluded from the results of these experiments?

1. Long-term exposure to high but well-tolerated concentrations of cigarette smoke (i.e., to optimal doses) induced epithelial changes in the larynx which are considered to be precancerous.* Results of our experiments give evidence that exposure to smoke, under the conditions and methods just described, is inducing tumors in the larynx.

2. During nose-breathing in smoke-exposed animals, the fast-moving smoke stream is slowed down considerably as it passes the narrow nasal orifice and enters the large nasal cavity (Fig. 16). Particles are deposited by impact on the intricate turbinal system of the hamster and the smoke flow is slowed down considerably (Fig. 17). Since deposition takes place on a large surface, there is obviously no possibility for the particles to settle and to concentrate at definite parts of any surface area. It is not likely, therefore, that the amount of smoke aerosols reacting per surface unit of the nasal area is high enough to induce tumors. The high respiratory frequency of the obligatory nose-breathing animals

*Final classification of the findings in the area of the larynx after long-term exposure to smoke, as well as final results of this experiment, will be reported elsewhere.

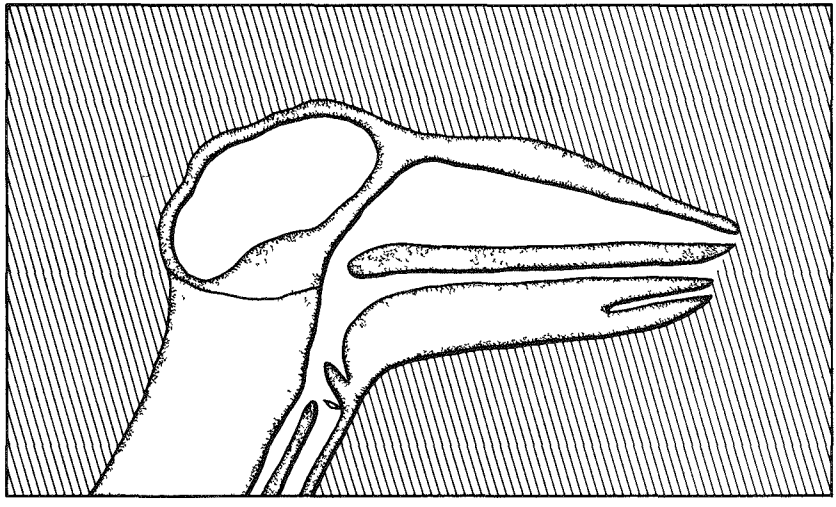


Fig. 16 – Rough model of the nasal and oral cavities of the hamster according to histological serial sections of particular head regions.

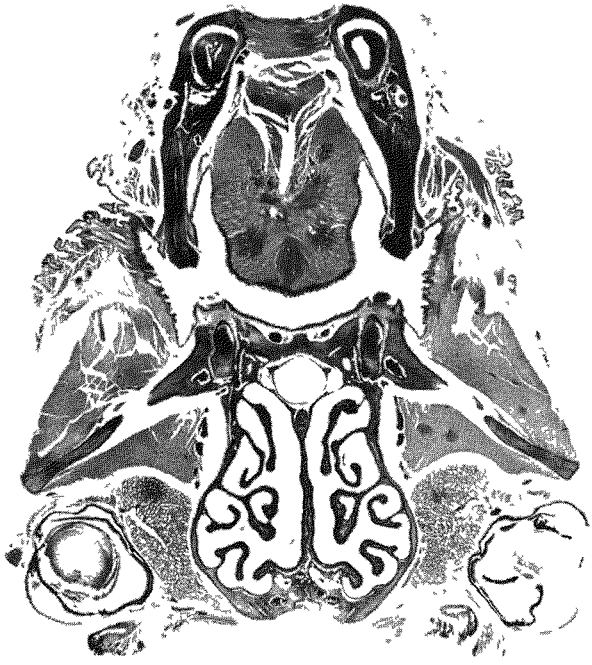


Fig. 17 – Cross section through head of hamster showing intricate turbinal system.

conducts the smoke stream in less than half a second to another target organ of impact, the larynx, producing a considerable slow-down of the smoke flow and leading to the deposition of the greatest amount of particulates on a small area. Reactions observed in the larynx may be explained by this mode of action.

This hypothetical explanation is supported by results of inhalation experiments with ^{14}C-labeled cigarette smoke (W. Dontenwill and H. P. Harke, unpublished observations). In comparing the relation of deposited ^{14}C-labeled particles in the smoke passage (mouth or nasal cavity, larynx, trachea, and lung), it could be demonstrated that the amount of deposited particles does not correspond with the surface area of the particular regions in the passage (Table 7). The amount of particulates deposited in the larynx is significantly increased, far more than would be expected for its small surface: impact and deposition of particles are considerable here. The amount of particles deposited in the larynx, per surface unit, makes the intensity of the histopathological findings in this area understandable.

What are the prospects of the results of our experiments?

1. This is probably the first time that the effect of total smoke on animals could be tested under controlled, standardized conditions and procedures; an improved model system for smoke inhalation studies has made this study possible.

TABLE 7

*Deposition of ^{14}C-Labeled Smoke Particles in Particular Regions of the Respiratory Tract**

Organ	Traced radioactivity (nCi)	Organ	Estimated radioactivity (nCi)	Deposition of particles (%)	Proportional area of the respiratory tract	Traced deposition in relation to the proportional area
Head and palate	6.11	Head, palate	5.5	37.4		
Tongue	0.41	Oral cavity in total	1.6	10.9		
Larynx	0.39				0.1–0.3	×561–187
Trachea	0.26	7.6 - traced		51.7	0.6	×62.3
Lungs	6.95				1000	×1
Total	14.12		14.7†	100		

*Cigarettes labeled with ^{14}C-1-*n*-hexadecan, data represent mean values from 10 animals, calculated from surface distribution in the head.

†The value of 14.7 contains 0 58 nanocuries as estimated from quantity of deposition in the nontraced oral cavity regions (calculated as to proportional area).

2. We are able to test whole smoke of modified cigarettes, which previously could be examined only as smoke condensate on skin or subcutaneous tissue. Only the investigation of whole smoke on the respiratory tract gives us a clear answer to the real activity.

REFERENCES

1. W Dontenwill and U. Mohr, Über Tracheal- und Bronchial-carcinome bei Goldhamstern nach Behandlung mit Diathylnitrosamin. *Klin. Wochschr.*, 39 (1961) 493.
2. W. Dontenwill and U. Mohr, Carcinome des Respirations-tractus nach Behandlung von Goldhamstern mit Diathylnitrosamin. *Z. Krebsforsch.*, 64 (1961) 305–312
3. W. Dontenwill, U. Mohr, and M. Zagel, Über die unterschiedliche Lungen-carcinogene Wirkung des Diathylnitrosamin bei Hamster und Ratte *Z. Krebsforsch.*, 64 (1962) 499–502.
4. W Dontenwill and U. Mohr, Experimentelle Untersuchungen zum Problem der Carcinomentstehung im Respirationstrakt. II Die Wirkung von Tabakrauchkondensaten und Zigarettenrauch auf die Lunge des Goldhamsters *Z. Krebsforsch.*, 65 (1962) 62–68.
5. J M Essenberg, Cigarette smoke and the incidence of primary neoplasm of the lung in the albino mouse. *Science*, 116 (1952) 561–562.
6. W. Dontenwill and B. Wiebecke, Tracheal and pulmonary alterations following the inhalation of cigarette smoke by the golden hamster. In, *Lung Tumours in Animals*, pp. 519–526, L. Severi (ed.). Division of Cancer Research, U. of Perugia, Italy, 1966.
7. W. Dontenwill, G. Reckzeh, and L. Stadler, Inhalationsexperimente mit Cigarettenrauch. *Beitr. Tabakforsch.*, 3 (1966) 437–447.
8. W. Dontenwill, G. Reckzeh, and L. Stadler, Berauchungsapparatur fur Laboratoriumstiere. *Beitr. Tabakforsch.*, 4 (1967) 45–49.
9. G Reckzeh, K. Rucker, H.-P. Harke, and W. Dontenwill, Untersuchungen zur Bestimmung der akuten und chronischen Toxizitat von Cigarettenrauch bei passiver Berauchung von Versuchstieren. *Arzneimittel-Forschung*, 2 (1969) 237–241

10 K Rucker, and W Dontenwill, Tierexperimentelle Untersuchungen über die Beeinflussung der Atmung während der Inhalation von Cigarettenrauch *Beitr Tabakforsch*, 4 (1967) 40–44
11 G Reckzeh and W Dontenwill, Effects of cigarette smoke on hamsters Experimental studies on skin temperature, body weight, leukocyte and erythrocyte counts *Arch Environ Health* 20 (1970) 7–15
12 W Dontenwill H P Harke, U Lafrenz, and G Reckzeh, Die Wirkung von Benzpyren, Zigarettenrauchkondensat und passiver Berauchung auf die Bildung der Zoxazolaminhydroxylase *Experientia*, 24 (1968) 714–715.
13 R M Welch, Y E Harrison, B W Gommi, P J Poppers, M Finster, and A H Conney, Stimulatory effect of cigarette smoking on the hydroxylation of 3,4-benzpyrene and the *N*-demethylation of 3-methyl-4 mono-methylaminoazobenzene by enzymes in human placenta *Clin Pharmacol Therap* 10 (1969) 100–109
14 P Kotin, I A Mitchell, and H L Falk, The role of polluted urban air and associated factors in the pathogenesis of respiratory tract cancer In, *Krebs – Dokumentation und Statistik maligner Tumoren*, pp 171–181, G Wagner (ed) F K Schattauer-Verlag, Stuttgart, 1968
15 W Dontenwill, H Elmenhorst, G Reckzeh, H-P Harke, and L Stadler Experimentelle Untersuchungen uber Aufnahme, Abtransport und Abbau cancerogener Kohlenwasserstoffe im Bereich des Respirationstraktes *Z Krebsforsch*, 71 (1968) 225–243
16 U Saffiotti, F Cefis, and L H Kolb, A method for the experimental induction of bronchogenic carcinoma *Cancer Res*, 28 (1968) 104–124
17 K M Herrold and L J Dunham, Induction of carcinoma and papilloma of the tracheobronchial mucosa in the Syrian hamster by intratracheal instillations of benzo[*a*]pyrene *J Natl Cancer Inst*, 28 (1962) 467–491
18 K M Herrold and L J Dunham, Induction of tumors in the Syrian hamster with diethylnitrosamine (*N*-nitrosodiethylamine) *Cancer Res*, 23 (1963) 773–777
19 M Kuschner, The J Burns Amberson Lecture The causes of lung cancer *Am Rev Respirat Diseases*, 98 (1968) 574–590
20 E L Wynder and D Hoffmann, *Tobacco and Tobacco Smoke Studies in Experimental Carcinogenesis* Academic Press, New York, 1967
21 H Druckrey and R Preussmann, Zur Entstehung carcinogener Nitrosamine am Beispiel des Tabakrauchs *Naturwissenschaften*, 49 (1962) 498–499
22 H Otto, Experimentelle Untersuchungen an Mausen mit passiver Zigarettenrauchbeatmung *Frankfurter Z Pathol*, 73 (1963) 10–23
23 J E Ash, M R Beck, and J D Wilkens, Tumors of the upper respiratory tract and ear In, *Atlas of Tumor Pathology*, Sec IV, Fasc 12–13, F 12–16, 1964

DISCUSSION

T. Crocker: I am very interested in the differential, metabolic effects of cigarette smoke exposure on rats, hamsters, and mice, in which greater survival was demonstrated in one species than in another You relate the longer life of some animals to metabolic competence for the destruction of nicotine Is there a correlation between the ability to survive, based on metabolic inactivation of nicotine, for example, and the degree of abnormality of tracheobronchial epithelia? Does an animal that survives longer, because of its metabolic protective mechanisms, exhibit greater or less epithelial abnormalities in the airways?

W. Dontenwill: There is not only a metabolic difference with respect to nicotine: we also measured the difference in hydroxylase activity and found that subcutaneous treatment with benzo[*a*]pyrene is able to induce zoxazolamin-hydroxylase [2-amino-5-chlorobenzoxazole hydroxylase]. Furthermore, we found a different adaptive enzyme production in different animal species: enzyme activity in hamsters is an order higher, whereas the enzyme system in rats is capable of higher inducibility.

Crocker: Was this difference in enzyme activity associated with greater or less degrees of epithelial abnormality?

Dontenwill: Well, I think one of the different reactions in animals, or in humans, with respect to different carcinogens, comes from the different enzyme activity. We found in hamsters, for example, a very different enzyme activity. The range of the difference is very high in one colony.

Y. Alarie: I would like to ask both you and Dr. Auerbach, what do you see in the bronchial lymph nodes of your animals exposed to cigarette smoke? You have presented a lot of histological slides on other areas, and I think Dr. Auerbach presented a few on the lymph node. What kind of specific change do you see there?

Dontenwill: I think the specific change is a brownish pigment in the lymph node, or a proliferation of the sinus epithelium. Brownish pigment, and black pigment, that is all. There are not more reactions.

N. Nelson: Did you want to add to that, Oscar?

O. Auerbach: We see brown pigmentation within the lymph nodes. We also see fat within them, as well as reticulum and lymphocytic hyperplasia. I would say that because of these changes the lymph nodes in the smoking dogs are about four to five times as large as those of the nonsmoking dogs.

A. Wehner: How long do you keep the animals in these restraining cages, at any given smoking period, and how many times a day can you safely do this without imposing an undue stress?

Dontenwill: In our smoking procedure we expose animals to smoke three times a day for 10 min each. Before calculating the amount of smoke inhaled by the animals, it is necessary to calculate the respiratory rate as well as the number of inspirations by the test animals at a given time.

In hamsters the respiratory rate is approximately 100 inspirations per minute, so when we expose them to smoke three times a day for 10 min each, this would amount to 3000 inhalations of smoke/air mixture for each hamster. There is no possibility during that time for the animals to inhale stale smoke, because the smoking chamber is filled every other second with fresh smoke, so that the hamsters are exposed continuously to fresh smoke during the inhalation period.

It is important to know the smoking cycles and procedures of other inhalation apparatus. In Dr. Homburger's machine, for instance, one cigarette is

smoked for 10 min. I don't know, however, how long the smoke of one puff stays in the smoking chamber. In case it stays for only 2 sec, each hamster will make approximately 330 inhalations when smoking 10 cigarettes a day. As we have seen in earlier experiments in which inhalation machines of other construction were used, animals are liable to stop breathing for 2 sec or more as soon as smoke enters the chamber, and then begin breathing normally as soon as the smoke is replaced by fresh air. This means that a stay period of smoke in the chamber of only 2 sec does not necessarily make the animals inhale the offered smoke. On the other hand, when smoke stays in the chamber for 1 min before the next puff is drawn, stale and aged smoke is inhaled by the animals nearly all the time.

M. Kuschner: We have been plagued for a long time by the inability to produce pulmonary changes with cigarette products. Dr. Nelson referred earlier in the conference to the hazards of poor experimental design, in the face of very strong epidemiologic evidence.

I first saw Dr. Dontenwill's results earlier this year in Lusanne with a group of people who were there for a UICC Committee Meeting on inhalation cancer. Looking at his changes in the larynx, I was convinced that these were, to date, the most impressive changes produced in the respiratory epithelium by exposure to cigarette smoke products. As a matter of fact, I thought it wouldn't take much more to convince me that these changes were already invasive carcinoma. I think that Dr. Dontenwill has been very modest in his claim as to the nature of these changes. All of us would feel a little happier, of course, if the changes extended beyond the larynx and eventually metastasized. However, I think we all feel they would if the animals were kept alive long enough.

I think these results demonstrate the fact that what we have been suffering from, up to now, has been (1) a dosage deficiency in our experimental techniques, and (2) a failure to use whole smoke, a very complex material, which permits the summation of the number of effects.

Nelson: Before we end the discussion I believe that Dr. Furst has several comments he would like to make about the factors to be considered in tobacco smoke inhalation studies.

A. Furst: For the past 15 years the Council for Tobacco Research – U.S.A., because of its interest in the problem of human smoking and health, has been concerned with the proper bioassay methods for tobacco smoke inhalation research. The scientific staff recommends that the following factors be considered when using an animal model.

First and foremost, meaningful data can only be obtained if the whole smoke delivered to the various parts of the respiratory tract of the animal is in the same physical and chemical state as that which reaches similar anatomical areas of the human during normal cigarette smoking.

In order to accomplish this, it is necessary to have a mechanical device – a smoking machine – that will simulate human smoking as closely as possible. This

device should be able to deliver pulsed amounts of smoke in a quantitative and reproducible manner. The machine should be so designed and constructed that it will be possible to measure accurately the time between two successive puffs, the time of the puff, the volume of the puff, the extent of the dilution with air, and the number of puffs per cigarette. Care must be taken so that the temperature of the burning tip does not exceed that reached when a human smokes a cigarette, nor should the cigarette be "smoked" beyond a minimum butt length.

The machine should have a port to permit sampling of the smoke stream for chemical or gas chromatographic analysis, if desired. For fractionating the whole smoke and to permit the bioassay of the gaseous phase, the smoking machine should be designed so that a Cambridge filter can be attached. For comparisons between laboratories, it is desirable that a reference-type cigarette be used, such as the one now being produced at the University of Kentucky.

The animal receiving the smoke should be placed so that only its breathing apparatus is in the mainstream of the smoke and no farther than the distance of the human nose to the main branching of the airways in human lungs. The animal used should be fully described as to age, genetic background, weight, nutritional state, physical condition, virus components, extent of surgical trauma, and the degree of stress to which it is subjected. Appropriate numbers of both sham-smoked and cage-held animals should serve as controls.

The end points sought include more than morphological alterations and the presence or absence of tumors. They also should include appropriate biochemical measurements and their analysis. Multivariate analysis should then be applied to the data obtained from the systematic observations of the controlled parameters relative to the smoking machine, the animal model, and the biological and biochemical measurements. By these means, it may be possible to interpret some of the biological effects resulting from exposure of animals to cigarette smoke. Past experiments, where the sole objective was the attempt to induce tumors, must be carefully and cautiously interpreted in light of today's knowledge.

URANIUM MINE AIR CONTAMINANTS IN DOGS AND HAMSTERS

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ABSTRACT

In response to growing national concern over the increased incidence of lung cancer in uranium miners, a program of daily exposures of experimental animals was undertaken to determine the biological effects of repeated chronic inhalation of several potentially carcinogenic uranium mine air contaminants. The possible synergistic or contributory actions of short-lived radon daughters, carnotite ore dust, and diesel engine exhaust in the development of respiratory tract pathology are being tested with six groups of 100 hamsters each. Each of the five experimental groups is exposed for 6 hr daily to aerosols of either: 30 working levels of radon daughters; 600 working levels of radon daughters; 600 working levels of radon daughters with 15 mg ore dust/m^3; diesel exhaust fumes; or diesel exhaust plus 600 working levels of radon daughters with ore dust. The remaining hamsters are being maintained as controls. — Because nearly all of the uranium miners that have developed lung cancer have histories of cigarette smoking, experiments with beagle dogs have been initiated to determine whether radon daughters and cigarette smoke inhalation may jointly cause respiratory tract damage. Three groups of 20 dogs each are receiving daily exposures to: the smoke of 10 cigarettes over a 16-hr period; cigarette smoking plus 4 hr of exposure to 600 working levels of radon daughters with 15 mg ore dust/m^3; 600 working levels of radon daughters with ore dust plus sham smoking. — The current status of these long-term multiple exposure studies is described.

The high incidence of lung cancer observed among uranium miners of the Colorado Plateau has focused national concern upon the working environment of these men, and in particular upon the components of the mine air that may be potential carcinogens. A recent report upon the etiology of this disease in underground uranium miners records an incidence of 62 cases among a total of 3414 men involved in the study, which is six times that observed in nonminers in this area.[1]

Current tabulations of the number of diagnosed cases of lung cancer in uranium miners indicate more than 150 cases, with carcinomas arising both in the hilar and peripheral regions.[2] Retrospective estimates have been made of mine air radioactivity levels in Saxony and Czechoslovakia during the last century, and comparison of cumulative exposures of miners with the extremely high incidence of observed lung cancer has led to the conclusion that the radioactive decay products of radon may be the major carcinogenic agent in the production of lung cancer in these men.[3-5] However, the Colorado Plateau miners are exposed to many other hazardous air contaminants, including blasting gases, ore dust, and diesel exhaust fumes from trackless mobile equipment.[6] The majority of the miners are also heavy cigarette smokers.[6] While some of the miners have received calculated total cumulative exposures of more than 6000 working level months (WLM),* others who have developed lung cancer have received much lower exposures. A recent review has emphasized the complicating factor of previous hard-rock mining experience in determining the cause and effect relationship of lung cancer in miners who received estimated exposures of only 100–400 WLM.[7]

In addition to the radioactive decay products of radon, the other airborne contaminants mentioned above may independently or synergistically increase damage to lungs. Siliceous uranium ore dust concentrations in present mines may reach nearly opaque cloud levels in some operations, and levels approaching 100 million particles/ft^3 were frequent in the many small mines operating on the Colorado Plateau in the late 1940's and early 1950's.[8] Diesel equipment has been in use in the mines since the early 1950's, and has produced heavy, oily soot collections on air samples taken in representative mine passageways.[9] Kotin *et al.*[10] have found greatly increased benzo[*a*]pyrene levels in exhaust from inefficient diesel engines under load; these operating conditions are very similar to those found in many of the uranium mines. Other exhaust constituents analyzed as aldehydes have been found to exist in mine air in concentrations high enough to be irritating, and nitrogen oxide levels must be routinely monitored.[11]

The possibility that cigarette smoking among U.S. uranium miners may play a major contributory role in the incidence of lung cancer has been repeatedly suggested. A cigarette-smoking uranium miner may have an eight-times greater risk of developing lung cancer than does a nonsmoking miner.[12] The Subcommittee on Radiation Exposure of Uranium Miners states that only two of the sixty-two deaths from lung cancer occurred in nonsmokers.[7] Based on the percentage of miners who smoked, if the deaths had occurred independently of smoking, eleven of these deaths should have occurred in nonsmokers, suggesting that radiation damage and the effects of smoking may be synergistic. The effects of cigarette smoking have been described by Auerbach[13] as loss of cilia,

*One working level month (WLM) is defined as 170 hr in an atmosphere containing 1.3×10^5 Mev of total potential alpha disintegrations per liter.

increased numbers of cell rows, and the appearance of atypical cells in the thickened epithelium leading to metaplasia and finally carcinoma *in situ* and invasive cancer in the bronchial epithelium. He also observed fibrosis, rupture of alveolar septa, and arterial wall thickening in the peripheral lung.[14] The disruption of normal cell type and structure throughout the respiratory tract by inhalation of cigarette smoke may augment the radiation damage from radon daughters by increasing the effective range of alpha particles, by damaging epithelium, and by affecting normal clearance mechanisms to permit localization of carcinogens at sites which may also receive concentrated radiation damage. The importance of repetitive injury and repair cycling in the induction of carcinogenesis and the necessity of studying the possible contributory action of several concomitant airborne hazards, as discussed by Kotin,[15] emphasizes the need for daily, lifetime exposures of experimental animals to determine the biological effects of mine air pollutants.

A program was set up to study the effects, in experimental animals under controlled conditions, of four potentially carcinogenic air contaminants to which uranium miners are exposed, i.e., radon and its daughters, cigarette smoke, uranium ore dust, and diesel exhaust fumes. It is believed that results from these studies will provide the basis for definitive research to properly assess the interrelationships of the several hazardous materials in mine atmospheres working concomitantly to cause damage to the respiratory epithelium, emphysema, fibrosis, and precancerous or cancerous changes in the lungs.

HAMSTER EXPERIMENTS

Hamsters were selected as the animal species for this study because they can be accommodated in large numbers, they are relatively free of chronic respiratory disease, and their susceptibility to development of pulmonary carcinoma following the intratracheal injection of such materials as benzo[*a*] pyrene on hematite ore has been demonstrated.[16]

The experimental design for the hamster studies is shown in Table 1. The first four groups of 100 hamsters each (50 males and 50 females) were first exposed at 12 weeks of age and are currently being exposed for 6 hr per day, 5 days per week, in order to study the effects of inhalation of two levels of radon daughters, with and without carnotite ore dust. Two groups of 100 hamsters each are being used to test the possible additive or synergistic effects of inhaled diesel engine exhaust.

Figure 1 shows one of the inhalation exposure chambers designed and fabricated for these studies. One-hundred hamsters are housed individually in stainless steel wire cages, and six wedges containing 17 such cages are inserted through self-sealing rubber doors in the 70-inch diameter chambers after constant radon daughter levels are reached. Aerosols introduced at the top of each chamber flow through the cages, around a baffle plate positioned 12 inches below the animals, and are exhausted at the bottom of the chamber.

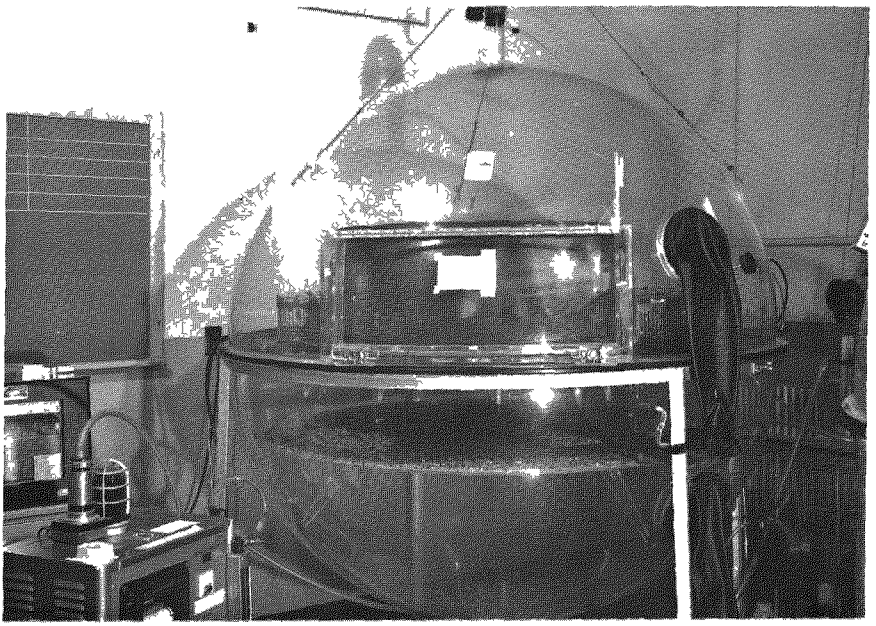

Fig 1 Typical chamber for daily exposure of 100 hamsters to constituents of uranium mine atmospheres

TABLE 1

Experimental Design Hamsters

	Exposure groups	Number of animals
1	Controls*	100
2	30 WL radon daughters	100
3	600 WL radon daughters	100
4	600 WL radon daughters with uranium ore dust	100
5	Diesel engine exhaust	100
6	Diesel engine exhaust plus 600 WL radon daughters with uranium ore dust	100

*Controls were sham-exposed under conditions identical to those used for the experimental groups

Concentration variations of less than 10% can be obtained with these chambers.

Figure 2 illustrates a 17-cage wedge constructed of stainless steel wire to provide minimal resistance of aerosol flow during exposures At the end of each daily 6-hr exposure, the cages are flushed with laboratory air After allowances

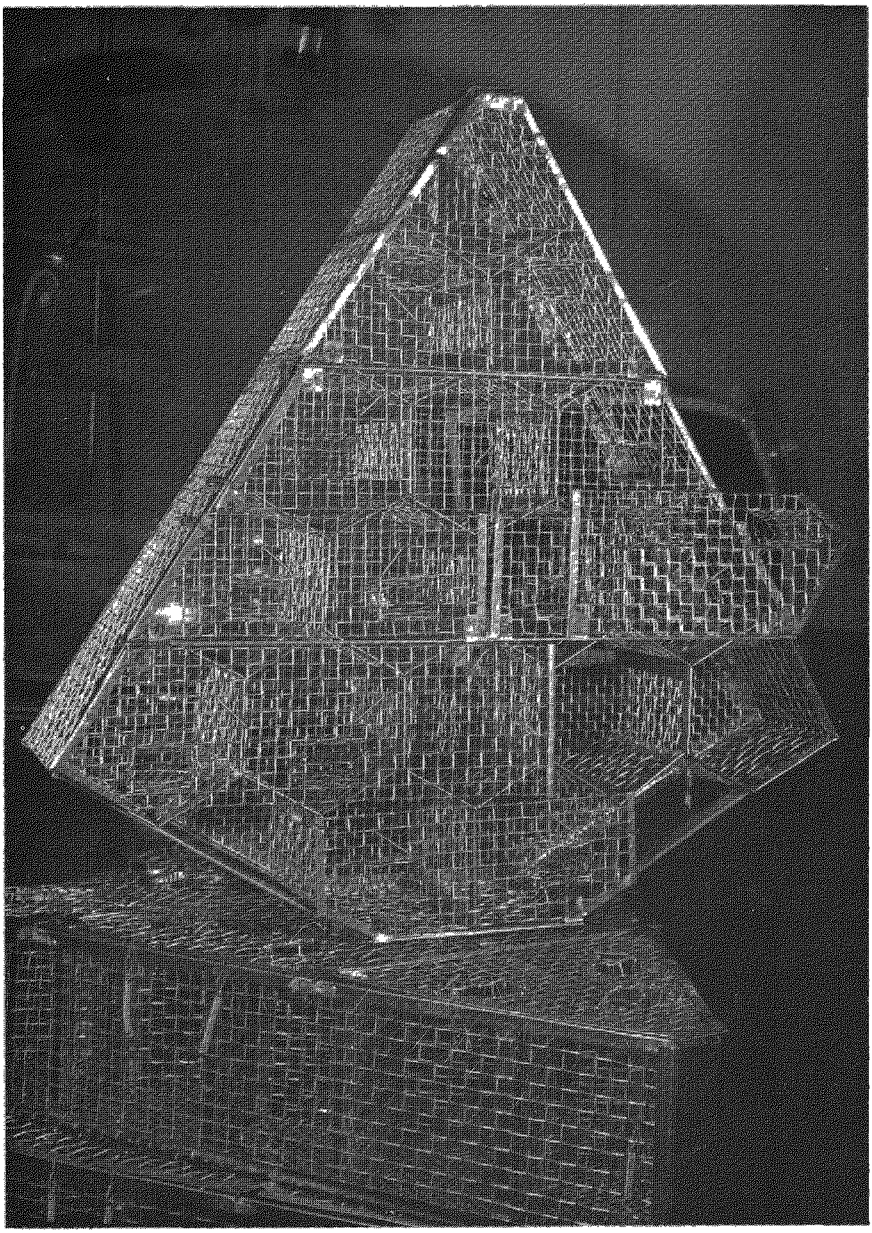

Fig. 2 – Wedge of 17 individual compartments for hamster inhalation exposures.

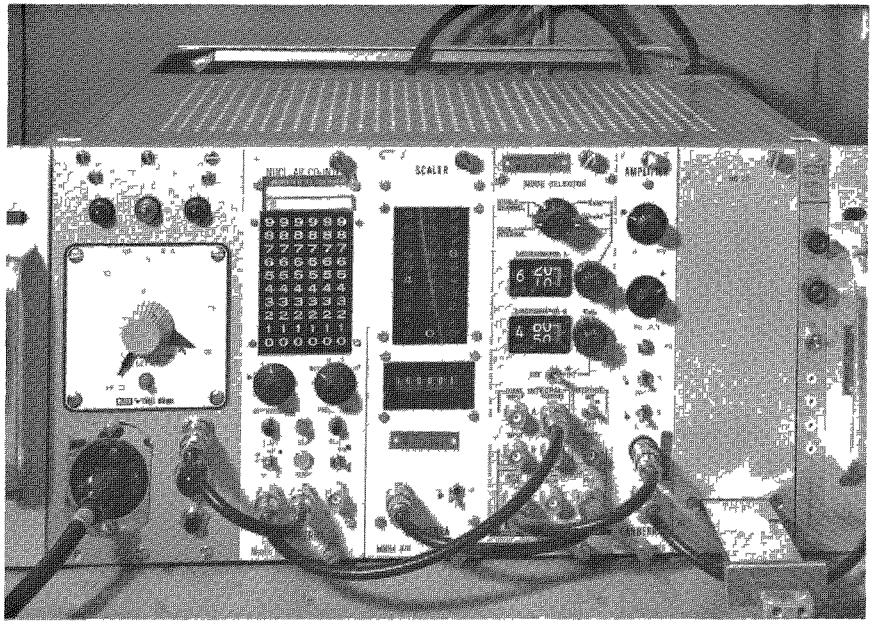

Fig. 3 – Portable radon daughter analyzer, utilizing membrane filter samples of chamber aerosols.

are made for physical decay of deposited radon daughters, the cages are removed and placed on bedding until the following day's exposure.

The solid-state analyzer developed for alpha-emitting radon daughter analyses is shown in Fig. 3. A timed 1-min sample is drawn from each chamber and inserted in the sliding drawer shown on the lower right. A surface barrier detector is used to monitor the discrete alpha energies of RaA and RaC′ at intervals separated by 20 min. The counting rates of these isotopes determine the concentration of all short-lived radon daughters in the chambers. Radon levels are monitored hourly in each exposure chamber and radon daughter levels are monitored daily. Fractional equilibrium levels of daughter radioactivity to radon are approximately 0.9, 0.5, 0.3 for RaA, RaB, and RaC–C′, respectively.

Carnotite ore dust, approximately 4% uranium by weight, is used for the ore dust exposures. Ore dust concentrations are monitored by membrane filter samplers followed by gravimetric and chemical analyses. The chamber concentrations are approximately 15 mg/m^3. Particle size analyses are made with Walkenhorst thermal precipitators followed by electron microscopy. Particle size distributions have count median diameters of approximately 0.4 μm. The relative humidity in the animal chambers is 50–70%. The temperature ranges from 70 to 76°F.

A diesel exhaust exposure system consisting of a new 35-hp diesel motor plus generator, resistor banks, cycle controller, and monitoring equipment has

been designed, fabricated, and installed to supply diesel exhaust to the animal chambers. Mine conditions of cyclic work load are simulated on the motor. The diesel exhaust levels will be limited to 40 ppm NO_x in the animal exposure chambers. The diesel exhaust will be monitored continuously during the 6-hr exposure period, 5 days per week. Particulate matter will be collected on membrane filters from the chamber during various motor work cycles. Selected samples will be analyzed to characterize the particle size distribution and concentration of the particulates.

Biweekly weight records are being maintained for each animal to compare effects between groups, to identify animals that are likely to die, and to obtain tissues for histopathology with minimum postmortem autolysis. Exposures of the hamsters will continue throughout their lifespans.

DOG EXPERIMENTS

The possible synergistic or additive effect of heavy cigarette smoking plus radon daughter and uranium ore dust inhalation in the initiation or development of lung cancers in uranium miners is strongly suggested by etiological studies. The experiments described below are designed to study the development of respiratory tract pathology resulting from inhalation exposures of dogs to these agents, both combined and separately, under controlled conditions.

The deposition of cigarette smoke particles in the respiratory tracts of small laboratory animals may be dissimilar from that in humans because of the much smaller diameters and lengths of air passages, greatly different respiration volumes and rates, and the general experimental practice of whole-body exposures. Few studies have been completed with an exposure regimen similar to that in heavy smokers, i.e., intermittent smoke exposure 16 hr/day, 7 days per week.

The present studies employ techniques that allow dogs to smoke 10 cigarettes per day by mouth inhalation and nose plus mouth exhalation. This pattern of smoking closely simulates human patterns.[17] Earlier studies showed that smoking by beagle dogs had little effect upon the clearance of submicron particles from the alveolar area of the lung,[18] but the effects of chronic cigarette smoking itself upon the nasopharynx, tracheobronchial, or pulmonary regions of the lungs have not been studied by use of a realistic simulation of human smoking. With these techniques and head-only exposures to radon daughters and uranium ore dust, the dogs should receive tracheal and lung exposures similar to man. Auerbach[19] has found that the acute histopathologic response of the lung of dogs exposed to cigarette smoke was similar to that observed in human smokers. The long lifespan of the dogs may permit tumors to develop, if a long latent period similar to that in man is required.

Sixty-nine beagle dogs were raised in our colony and trained in preparation for exposures as outlined in Table 2. Each dog was thoroughly trained to the cigarette-smoking apparatus, which is shown in Fig. 4. This U-shaped module

TABLE 2

Experimental Design· Dogs

Exposure groups	Number of animals
1. 600 WL radon daughters with uranium ore dust	20
2. Cigarette smoke	20
3. Cigarette smoke plus 600 WL radon daughters and uranium ore dust	20
4. Controls*	9

*Controls were sham-exposed under conditions identical to those used for the experimental groups.

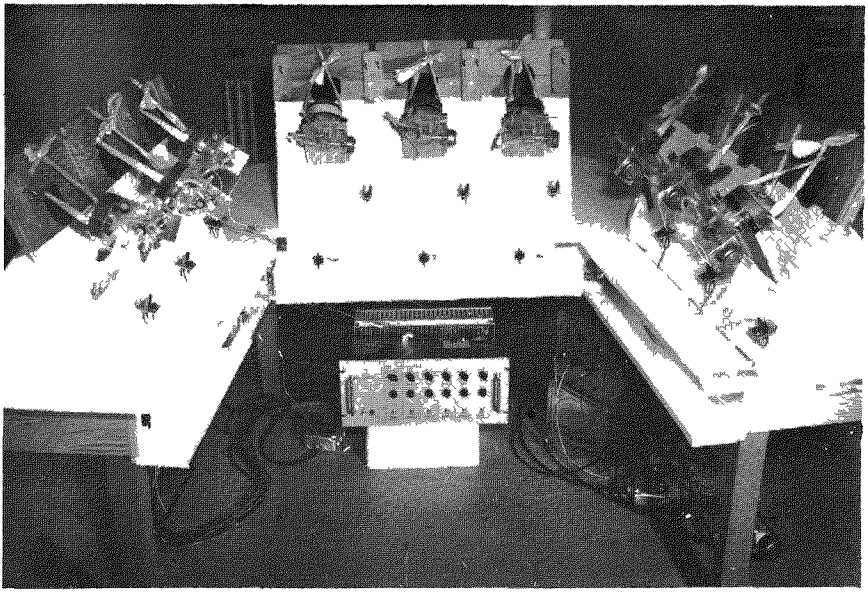

Fig. 4 – Nine-dog module for exposures to cigarette smoke. Each animal's breath is individually monitored and valves are activated on demand.

was developed to permit simultaneous exposure of dogs to cigarette smoke in a manner analogous to human exposures. Each dog's respirations are monitored by means of the pressure drop across an in-line venturi, and these changes are transmitted by pressure switches to an electronic counter and reset system. The ratio of breaths drawn through the cigarette to fresh-air breaths can be set to regulate the quantity of smoke inhaled per cigarette. As the selected number of breaths is reached by a particular dog, a trigger circuit activates a rotary solenoid

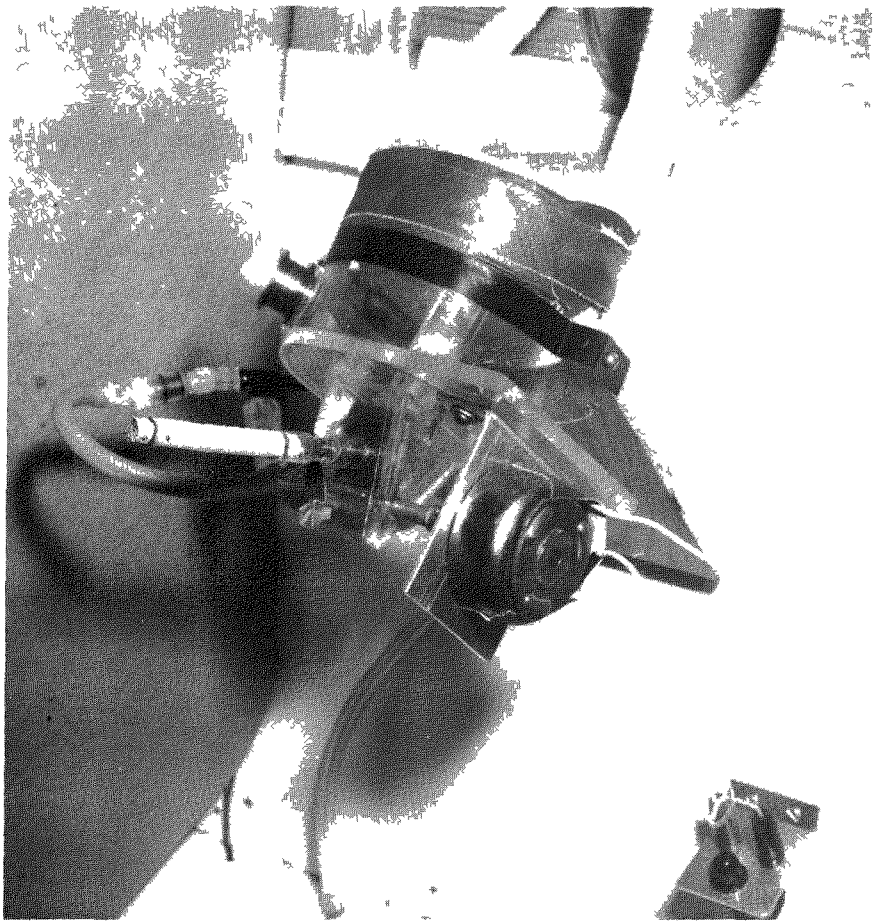

Fig. 5 – Smoking mask for individual dogs, showing inhalation tube, rotary solenoid, and monitor light

that closes the fresh air orifice, thus requiring air to be drawn through the lighted cigarette. The next respiration releases the solenoid and resets the count-recording circuit. A more detailed view of the smoking mask developed for these exposures is shown in Fig. 5. A padded blade attached to the rotary solenoid shaft admits either air (bottom inlet) or smoke through a tube positioned in the dog's mouth. A visual check of operation is provided by a signal light that glows when smoke is being inhaled. Circular, one-way rubber valves serve to allow exhalation either through the nose (top of mask) or mouth (valve located between pressure-change conducting tubes). Figure 6 shows three dogs receiving cigarette smoke simultaneously.

Groups of 10 dogs each are exposed to radon daughters and ore dust in lucite exposure chambers such as that shown in Fig. 7. The use of two of these

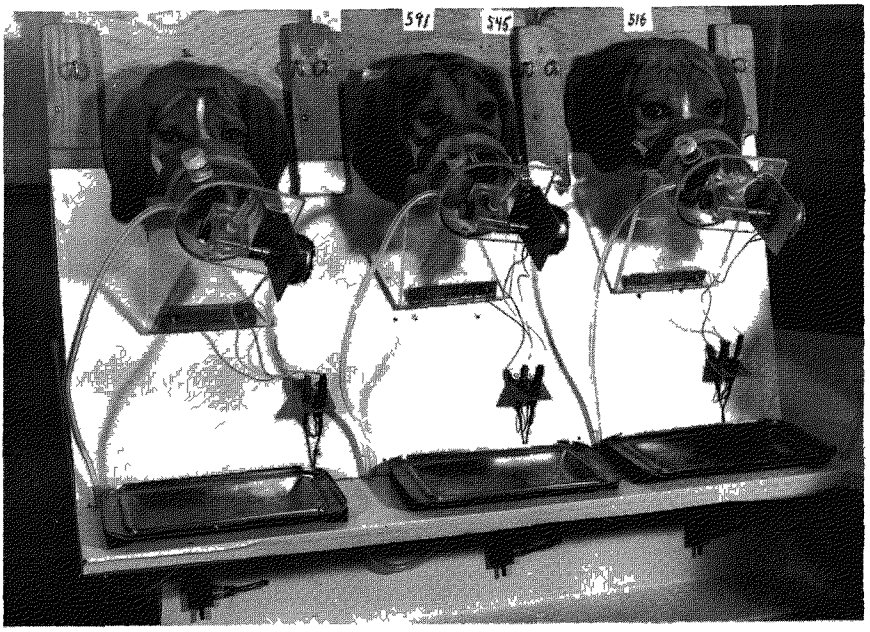

Fig. 6 – Simultaneous exposure of beagle dogs to cigarette smoke. Animals are trained to accept the mask and exposure regimen.

chambers allows 20 dogs to be exposed simultaneously. Aerosol is introduced at the top of the 6-ft high, octagonal chamber, and is exhausted around a baffle plate near the floor of the unit so that the flow of aerosol is channeled past the dog's heads. Each animal is placed in a separate exposure box with an attached exhaust system that draws air around the dog for body cooling and removal of any aerosol which may escape around the circular neck seal. Only the head of the dog protrudes into the chamber during exposure. A vertically sliding door seals the opening for each dog when the individual boxes are removed for cleaning. Radon monitoring, radon daughter sampling, and aerosol particle sizing are performed with instrumentation identical to that employed in the hamster studies.

The biological effects produced by the chronic inhalation of radon daughters plus ore dust and/or cigarette smoking will be identified from physical examinations including respiratory rate and volume, body weight, hematology, clinical chemistry, and thoracic radiographs. Histopathologic examinations of all respiratory and systemic tissues will be made after the first few months of exposure at sacrifice of one dog from each group. This dog will be replaced with a new dog in the exposure regimen. The respiratory tract will be studied for hyperplastic, metaplastic, or neoplastic changes in the tracheobronchial, bronchiolar, and alveolar epithelium, and for other possible changes such as emphysema and fibrosis. The effects produced in these animals will be compared with the

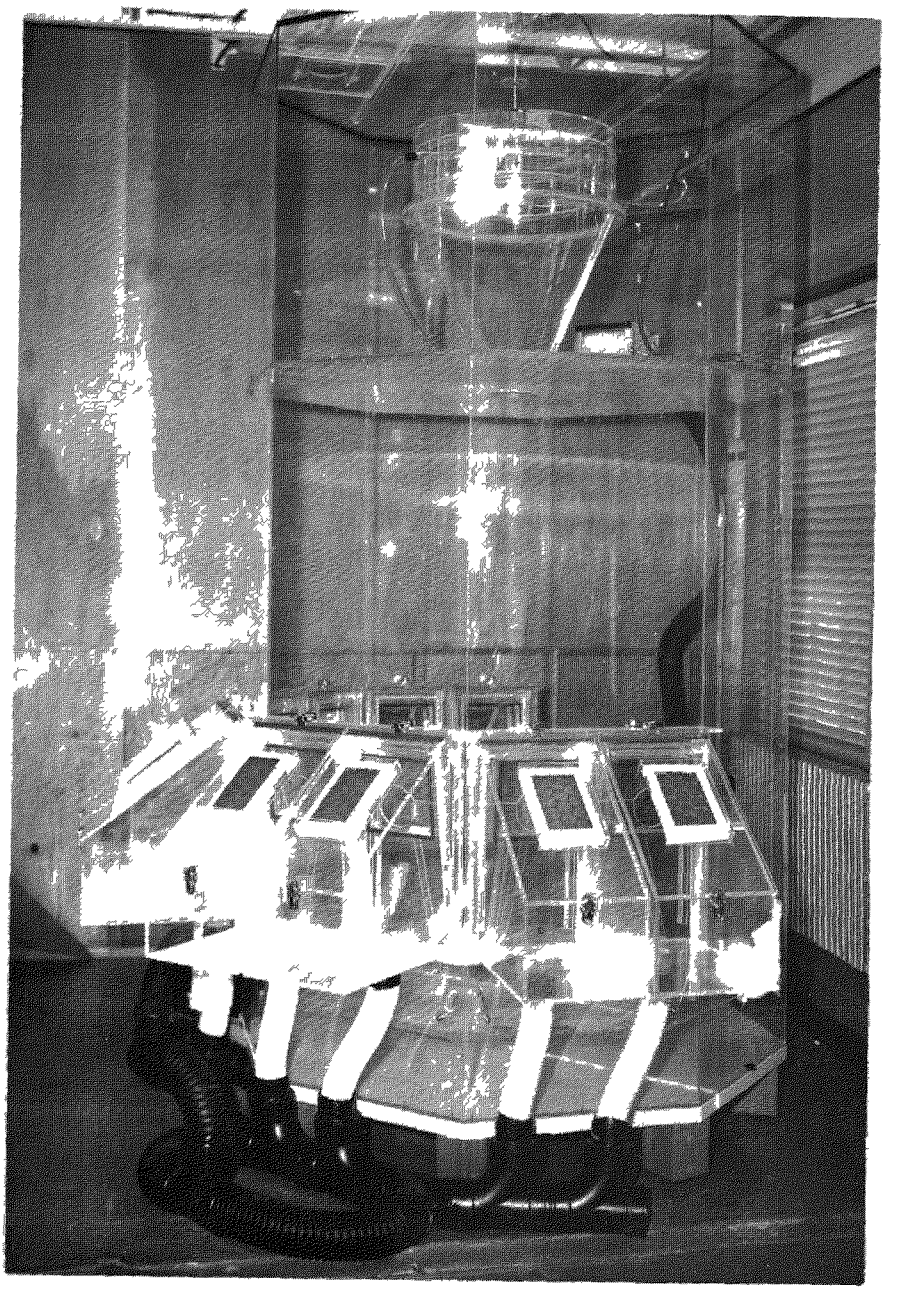

Fig 7 – Exposure chamber plus ventilated dog-holding boxes for simultaneous exposure of 10 dogs to radon daughters with uranium ore dust

lung pathology observed in uranium miners. Lung washings will be taken from two dogs in each group for comparison with sputum samples, lung washings, and autopsy samples of uranium miners. Seventeen dogs in groups 1, 2, and 3 will be maintained to study long-term effects.

RESULTS

Exposures of the 30-WL and control groups of hamsters have been underway for nearly 12 months. At 6–9 months after the start of daily exposures, the lungs of sacrificed animals showed some congestion as well as slight thickening, edema, and early hyalinization of the interalveolar septa. There was a slight amount of emphysema in the subplural regions.

After 6 months of daily exposures to 600 WL, the lungs of sacrificed hamsters showed a slight edema associated with the alveolar septa and small areas of peripheral emphysema (Fig. 8), with a moderate inflammatory reaction involving mononuclear and polymorphonuclear cells. Correspondingly, the lungs of animals exposed to 600 WL plus carnotite ore dust at 15 mg/m^3 for 6 months showed significant areas of alveolar septal breakdown with resulting emphysema.

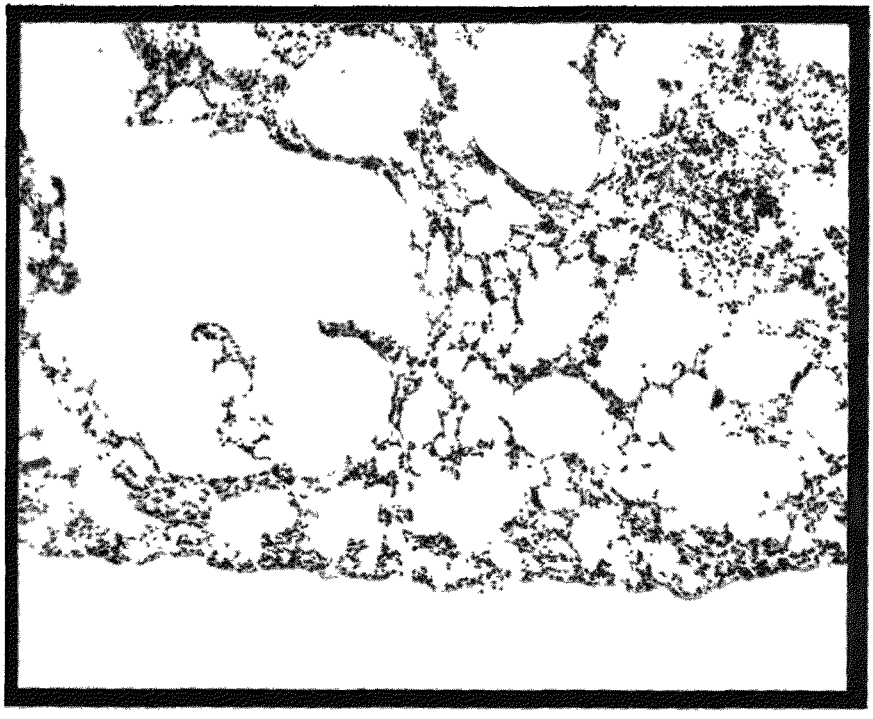

Fig. 8 – Lung section from a hamster at 6 months after start of daily exposures to 600 WL. Alveolar septal breakdown and emphysema are shown. H & E; 64X.

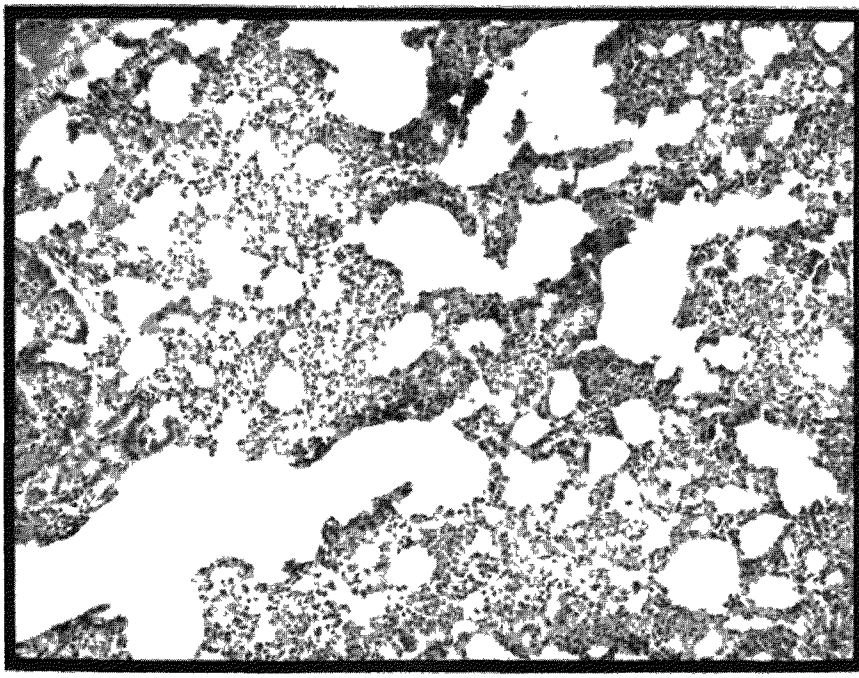

Fig. 9 – Lung section from a hamster at 6 months after start of daily exposures to 600 WL plus carnotite ore dust. Severe chronic pneumonitis is demonstrated. H & E; 64X.

Some edema and hyalinization of the interalveolar septa, and dilation and congestion of the vasculature system, including the smaller capillaries, was observed. Foreign body, giant cells were present and there was obliteration of many alveolar spaces. There was a severe generalized interstitial pneumonitis in most animals in this exposure group (Fig. 9). Less severe pneumonitis has been observed in some hamsters from all groups, including controls, that died or were sacrificed when death was imminent with signs of "wet-tail" syndrome. These observations must be regarded as preliminary; the final interpretation of these changes related to the exposure regimen will require detailed histopathological examination of all animals in these studies.

The training of beagle dogs for exposures to cigarette smoking and/or inhalation of radon daughters with uranium ore dust has been completed, and life-span daily exposures to these potentially carcinogenic agents have begun.

ACKNOWLEDGEMENTS

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Commission, and the National Institute of Environmental Health Sciences, Department of Health, Education, and Welfare.

REFERENCES

1. F. E. Lundin, Jr., J. W. Lloyd, E. M. Smith, V. E. Archer, D. A. Holaday, Mortality of uranium miners in relation to radiation exposure, hard-rock mining and cigarette smoking – 1950 through September 1967. *Health Phys.* 16 (1969) 571–578.
2. G. Saccomanno, personal communication, 1969.
3. A. Perchan and H. Sikl, Cancer of the lung in miners of Joachimsthal. *Am. J. Cancer,* 16 (1932) 1681.
4. C. G. Stewart and S. D. Simpson, The hazards of inhaling radon-222 and its short-lived daughters Consideration of proposed maximum permissible concentrations in air. In, *Radiological Health and Safety Mining and Milling of Nuclear Materials,* Vol. I, pp. 333–357. I A.E.A , Vienna, 1964.
5. R. D. Evans, Radiation protection of uranium miners. In, *Hearings on Safety and Health Standards.* Bureau of Labor Standards, U.S. Dept. of Labor, Washington, D.C , November 20, 1968
6. Federal Radiation Council, Guidance for the Control of Radiation Hazards in Uranium Mining, Staff Report No. 8, Revised Superintendent of Documents, U.S. Govt. Printing Office, Washington, D.C., September 1967
7. Federal Radiation Council, Radiation Exposure of Uranium Miners, Report of Advisory Committee from the Division of Medical Sciences, National Academy of Sciences – National Research Council – National Academy of Engineering, Washington, D.C., August 1968.
8. P W. McDaniel, New York City Health and Toxicology Department, personal communication, June, 1968
9. H. E. Palmer, R. W. Perkins, and B. O. Stuart, The distribution and deposition of radon daughters attached to dust particles in the respiratory system of humans exposed to uranium mine atmospheres. *Health Phys* , 10 (1964) 1129–1135.
10. P Kotin, H L. Falk, and M. Thomas, Aromatic hydrocarbons. III. Presence in the particulate phase of diesel-engine exhausts and the carcinogenicity of exhaust extracts. *A.M A. Arch Ind. Health,* 11 (1955) 113–120.
11. D. A. Holaday, personal communication, March 1968.
12. R. D. Evans, On the carcinogenicity of inhaled radon decay products in man (CORD). In, *Radiation Exposure of Uranium Miners, Hearings before the Subcommittee on Research, Development, and Radiation of the Joint Committee on Atomic Energy, Congress of the United States,* On the carcinogenicity of inhaled radon decay products in man, Part 2, p. 1199. Washington, D.C., 1967.
13. O. Auerbach, A. P. Stout, E. C. Hammond, and L. Garfinkel, Changes in bronchial epithelium in relation to cigarette smoking and in relation to lung cancer. *New Engl. J. Med.,* 265 (1961) 253–267
14 O. Auerbach, A. P. Stout, E. C. Hammond, and L. Garfinkel, Smoking habits and age in relation to pulmonary changes. *New Engl J Med.,* 269 (1963) 1045–1054.
15. P Kotin, Carcinogenesis of the lung Environmental and host factors. In, *The Lung,* International Academy of Pathology Monograph No. 8, pp. 203–225, A. A. Liebow and D. E. Smith (eds.). Williams and Wilkins Co., Baltimore, Maryland, 1967.
16. U. Saffiotti, F. Cefis, and L. H. Kolb, A method for the experimental induction of bronchogenic carcinoma. *Cancer Res.,* 28 (1968) 104–124.
17. W. J. Bair, N. S. Porter, D. P. Brown, and A. P. Wehner, Apparatus for direct inhalation of cigarette smoke by dogs. *J Appl. Physiol.,* 26 (1969) 847–850.

18. W. J. Bair and J. V. Dilley, Pulmonary clearance of $^{59}Fe_2O_3$ and $^{51}Cr_2O_3$ in rats and dogs exposed to cigarette smoke. In, *Inhaled Particles and Vapours,* II, pp. 251–271, C. N. Davies (ed.). Pergamon Press, Oxford/New York, 1967.
19. O. Auerbach, personal communication, 1968.

DISCUSSION

D. Hoffman: Did I read your slide [Fig. 5 in text] correctly? The mouths of these dogs are closed, is that correct?

B. O. Stuart: A small tube goes down about so far into his mouth, and he inhales through it, right through the center of the mouth. He inhales through the mouth and exhales through the nose or the mouth – all through one-way valves.

O. G. Raabe: I would like to know what size distributions you are using for your ore dust, and if you will tell us about the state of attachment of the radon daughters to the dust.

Stuart: The count median diameter of a particle is 0.3 to 0.4 μm. Standard geometric deviation is something approaching 2.0.

As to the percent attachment, I don't have any direct figures, but extrapolating from Dr. D. A. Morken's work in Rochester, with simple introduction of unfiltered ordinary room air, I believe that unattached radon daughters are no more than a few percent.

N. Nelson: What is the state of equilibrium of your radon daughters?

Stuart: Using the concentration of radon as a reference, the fractional levels are: for radium A about 0.9, for radium B about 0.5, and for radium C–C′ about 0.25 or 0.3.

PATHOLOGICAL EFFECTS IN THE RAT AFTER REPETITIVE EXPOSURE TO $^{152-154}$EUROPIUM

87-530

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There are at present no reports which indicate that inhaled beta-gamma emitters are carcinogenetic – although those implanted or given intratracheally, among them ^{103}ruthenium, ^{106}ruthenium, ^{32}chromium, ^{198}gold, ^{59}iron, ^{32}barium, and ^{144}cerium, do produce neoplasms in the experimental animal. Of the alpha emitters, only polonium and plutonium have been shown to be effective after inhalation by the rat and dog, respectively.

In order to test the cancerogenic potential of inhaled beta-gamma emitters, one of the rare earths, $^{152-154}$europium, which has a wide spectrum of both beta and gamma energies, was given by inhalation to three groups of rats at levels ranging from 0.71 to 31.0 $\times$ 10^{-6} μCi/ml air. A fourth group was exposed to equivalent amounts of stable europium, and still others were set aside as controls breathing room-air only.

The animals were females of the Sprague-Dawley strain, and no attempt was made to render them pathogen-free, except for the administration of polyotic, an antibiotic, in the drinking water for a few weeks prior to use.

Animals were exposed repetitively, 7 hr a day, 5 days a week for 24 weeks, and were sacrificed serially for activity and dose determinations. These rats were also observed grossly and histopathologically. In the longer term, moribund animals were observed for injury, particularly to lung tissue, and dead animals were examined if tissue autolysis was minimal.

Figure 1 represents the activity concentration both in the whole body and the lung at the lowest and highest aerosol levels. Figure 2 shows the accumulated dose in rads, calculated on the basis of isotope content per gram of tissue. Table 1 summarizes the pertinent findings for each of the exposure levels.

It should be noted that none of the animals were completely free of some pulmonary pathology. There were a large number of fibroadenomas of the skin

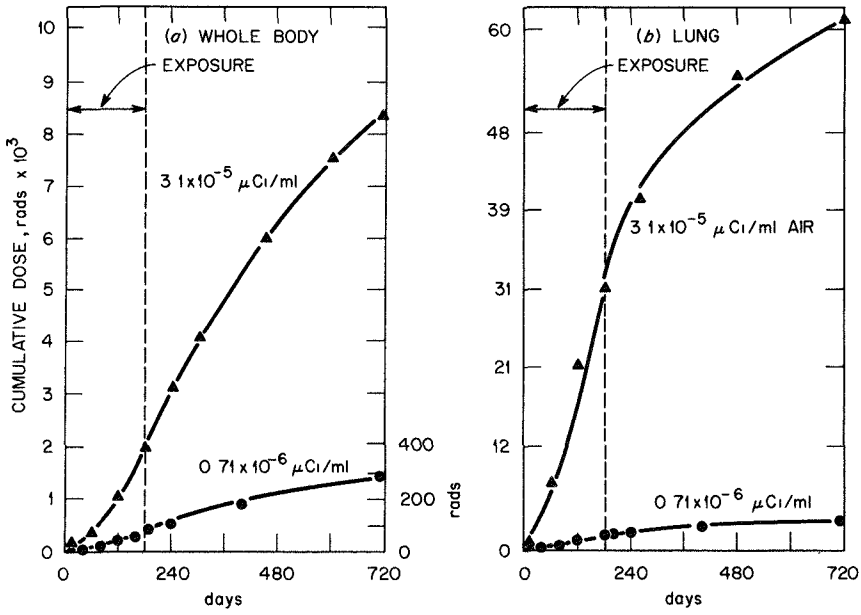

Fig 1 – Activity concentration after exposure to $^{152-154}$europium aerosols [From H L Berke *et al Intern J Radiation Biol* 14 (1969) 561]

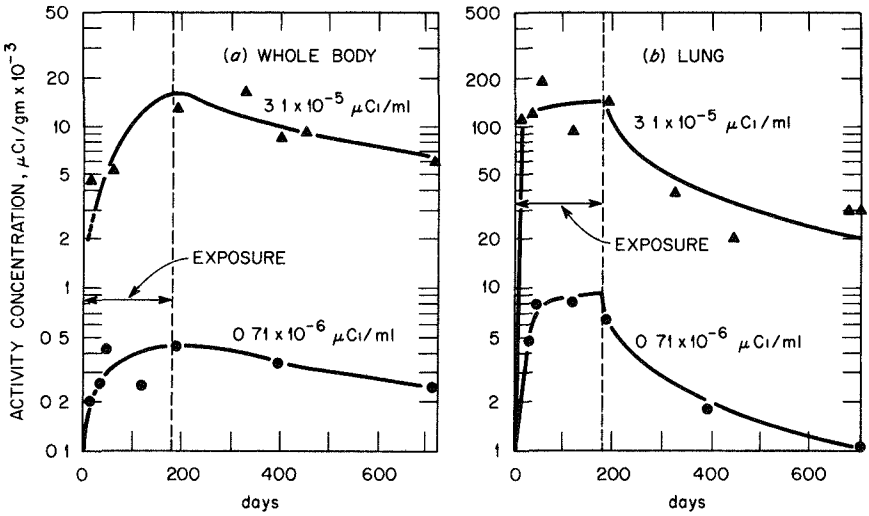

Fig 2 – Cumulative radiological dose of retained $^{152-154}$europium [From H L Berke *et al Intern J Radiation Biol* 14 (1969) 561]

TABLE 1

Summary of Pathological Findings in Rats Exposed Repetitively to Europium

	$^{152-154}$Europium (μCi/ml air)							
	0.7×10^{-6}		1.9×10^{-6}		3.1×10^{-6}		Stable Eu	
	C*	E†	C	E	C	E	C	E
No significant pathology	0	0	0	0	0	0	0	0
Pulmonary adenoma	0	0	0	0	0	0	0	0
Skin fibroadenoma	1	1	0	0	0	1	1	0
Pneumonia	0	2	1	3	0	1	1	1
Pneumonitis (slight)	0	0	0	2	0	0	0	0
Chronic inflammation (mild)	0	0	0	0	0	0	0	1
Large and/or confluent granulomas	0	2	1	1	1	1	0	1
Granulomas with fibrosis	0	2	0	0	1	0	0	0
Marked chronic inflammation	3	7	2	7	1	1	1	3
Moderate chronic inflammation	0	2	0	0	1	1	0	0
Multiple abscesses	2	7	4	9	1	6	7	4
Bronchiectasis	3	10	2	8	2	2	0	3
Totals	9	33	10	30	7	13	10	13

*Control.
†Experimental

in the subcutaneous and adnexal structures. These varied in appearance, with some of the more fibrous ones having a glandular or adenamatous appearance. Many were undoubtedly of mammary gland origin, were histologically benign, and showed no evidence of metastasis.

A considerable incidence of severe chronic inflammatory changes and lung abscesses were evident in a majority of the animals, both exposed and in the control group, suggesting the presence of communicable respiratory infection. The role of exposure to the radioisotope in the pathogenesis of the lesion is difficult to evaluate

The salient point is the complete absence of pulmonary neoplasias, which raises the question of the relative effectiveness of beta-gamma emitters in tumor production when given in aerosol form. It is possible that larger amounts of the beta-gamma emitters, perhaps lower energies for greater tissue effect, and other species more susceptible to lung tumors may result in observation of these pulmonary tumors.

The highly disperse form in which the inhaled isotopes of submicronic size are deposited may be a factor of importance in the etiology of the disease process. It may be, speculatively, that increased deposition will also be ineffective in the sense that the life-span of the animal will be markedly reduced by factors other than those of the lung. These are problems which should lend themselves to further study. The factor of mucociliary clearance rates is perhaps also deserving of greater consideration in inhalation pathologies.

DISCUSSION

R. O. McClellan: Dr. Berke, you noted the lack of reported cases of lung neoplasms produced by inhaled beta-emitting radionuclides. In view of this I would like to mention some recent work with inhaled ^{144}Ce oxide in the rat, performed in our laboratory by Mrs. Randi Thomas in collaboration with the late Dr. J. K. Scott and Dr. Tom Chiffelle.

Two groups of rats (low level and high level) were studied throughout their life-spans. In the high-level group of 23 rats, which had an average initial lung burden of 170 μCi and cumulative radiation doses to the lung of up to 2500 rads, a total of four squamous cell carcinomas were observed.

We have in progress a large study involving beagle dogs given single, brief inhalation exposures to ^{144}Ce incorporated into fused clay. In this experiment dogs with the highest initial lung burden died at about 6 months postexposure, and showed severe pulmonary fibrosis – changes similar to those reported by Dr. Berke.

We have a large number of dogs surviving at lower levels, but I am sure that they are prime candidates for the ultimate development of neoplastic changes.

N. Nelson: I believe that ^{144}Ce was used by Herman Lisco in one of the first instances of tumor production by inhalation exposures to radiation.

EFFECT OF BENZO[*a*]PYRENE ON HAMSTER, RAT, DOG, AND MONKEY RESPIRATORY EPITHELIA IN ORGAN CULTURE

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In the discussion following Dr. Saffiotti's presentation [see these *Proceedings*, p. 53] I stated briefly that I had found roughly similar cellular and histologic alterations produced by comparable concentrations of benzo[*a*]pyrene in rodent, canine, and primate respiratory epithelia in organ culture. This comment was made during a discussion of the comparability of any one animal model with the human respiratory system during inhalation exposure to toxins or carcinogens. At that moment, the discussion was directed by Dr. Kilburn toward the anatomic and aerodynamic properties of airways in different orders of mammals. In agreeing with Dr. Kilburn's desire to examine comparability among animal and human airways, I had wished to add emphasis to the comparability of cellular, metabolic, and nutritional characteristics of respiratory tissues from human and animal models for inhalation carcinogenesis. These elements in the model are not more or less important than respiratory function and anatomy. Rather, all these factors – as well as immunologic, genetic, and other characteristics of the animal or human system – are involved in the effect of inhaled materials.

The data dealing with comparisons among animal tissues are given here chiefly to emphasize cellular responses to benzo[*a*]pyrene.

Whole suckling hamster tracheas, whole bronchial tubes dissected from lungs of late fetal dogs and rhesus or cynomolgus monkeys, and tracheal plaques from the dogs and monkeys were laid on a rayon mesh on the surface of a clotted medium containing chicken plasma, embryo extract, and serum. Benzo[*a*]pyrene (BaP) was added to the serum in an acetone solution, so as to give final continuous concentrations of 7 to 15 μg/ml in medium. Explants were changed to new medium every 2 to 3 days and were incubated at 37°C in a box gassed with oxygen and carbon dioxide to give final concentrations of 50% O_2 and 2%

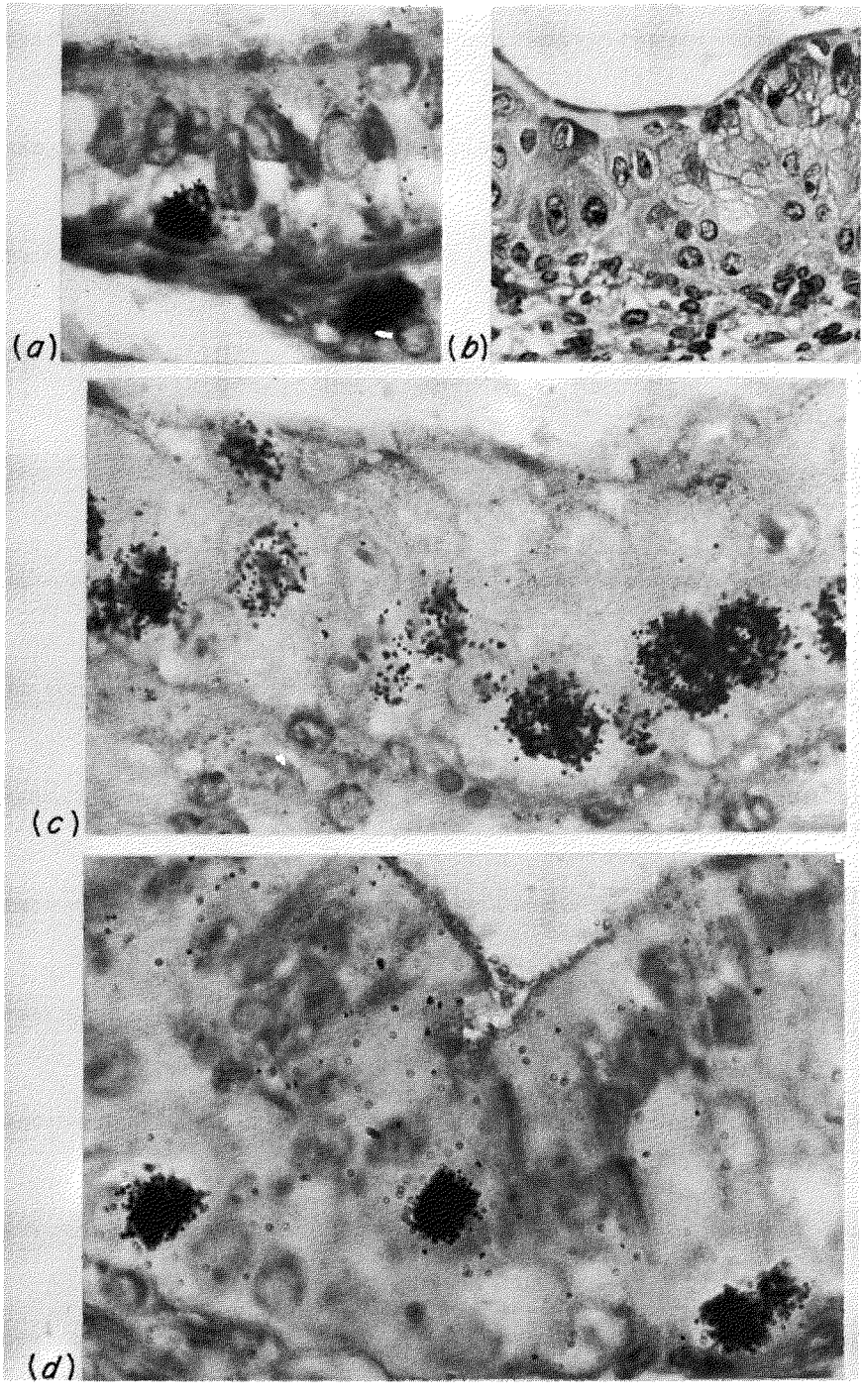
(a)
(b)
(c)
(d)

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Fig. 1 – Epithelia of suckling hamster tracheas treated with: (a) vitamin A, 10 IU, 15 days culture. The labeling rate in this well-differentiated epithelium is 1.9%. Cartilage staining is moderately reduced. Connective tissue between cartilage and epithelium is normal. 1000X. (b) BaP, 10.5 μg/ml, plus citral, 2 m*M*, 8-day culture. The two agents together produce a more marked squamous metaplasia at an earlier time than does BaP alone. Cartilage matrix is reduced in amount and mucopolysaccharide content, and inner connective tissue is proliferating. 400X. (c) As for (b); the labeling rate in this section of the explant is 21.7%, while the mean from a series of sections was 10.7%. 1000X. (d) Vitamin A, 20 IU, plus BaP, 10.5 μg/ml, 15-day culture. The labeling rate in this columnar epithelium with basal cell hyperplasia is 9.2%, indicating that the replicative stimulation of BaP is not inhibited by vitamin A in this instance. In other explants, however, labeling rates were as low as 1.7%. Cartilage is destroyed, and inner connective tissue is mixed with chondrocytes. Outgrowth of connective tissue is suppressed, a characteristic effect of BaP that is not inhibited by vitamin A. 1000X. (From T. T. Crocker and L. L. Sanders, *Cancer Res.*, in press, 1969)

CO_2. After 8 to 15 days of maintenance, explants were incubated with tritiated thymidine for 30 min, then processed for histology, electron microscopy, and autoradiography.

Vitamin A dissolved in ethanol was added to the serum in some experiments; final concentrations of the vitamin in media were 10 to 20 IU/ml. Most vitamin A-treated and control explants retained columnar epithelia with low levels of thymidine incorporation (Fig. 1a). Benzo[*a*]pyrene alone, or in combination with citral, a vitamin antagonist, produced some squamous metaplasia (Fig. 1b) but more often produced pleomorphic cells in a dysplastic epithelium with low to high labeling (Fig. 1c). Vitamin A plus BaP produced high, differentiated epithelium; hence vitamin A inhibited abnormal differentiation produced by BaP. Labeling levels were lower with BaP plus 20 IU/ml vitamin A (Fig. 1d) than with BaP plus 10 IU/ml vitamin A. This represented a dose related partial inhibition of the proliferative effect of BaP on hamster tracheal epithelium by vitamin A.

Electron microscopy of rat tracheas exposed to BaP for 11 days revealed loss of columnar differentiation. Autophagic vacuoles, desmosomes, tonofilaments, and cellular interdigitations corresponding with squamous metaplasia were also found (Figs. 2a–c).

Fetal dog tracheal epithelium was fairly well maintained in control culture (Fig. 3a) and developed squamous metaplasia with high replicative activity upon exposure to BaP (Fig. 3b) at concentrations and times effective in hamster trachea.

Fetal monkey tracheobronchial epithelium was well differentiated in control cultures (Fig. 4a) but after treatment with BaP became poorly differentiated, though columnar (Fig. 4b), or was converted to a few layers of pleomorphic cells (Fig. 4c).

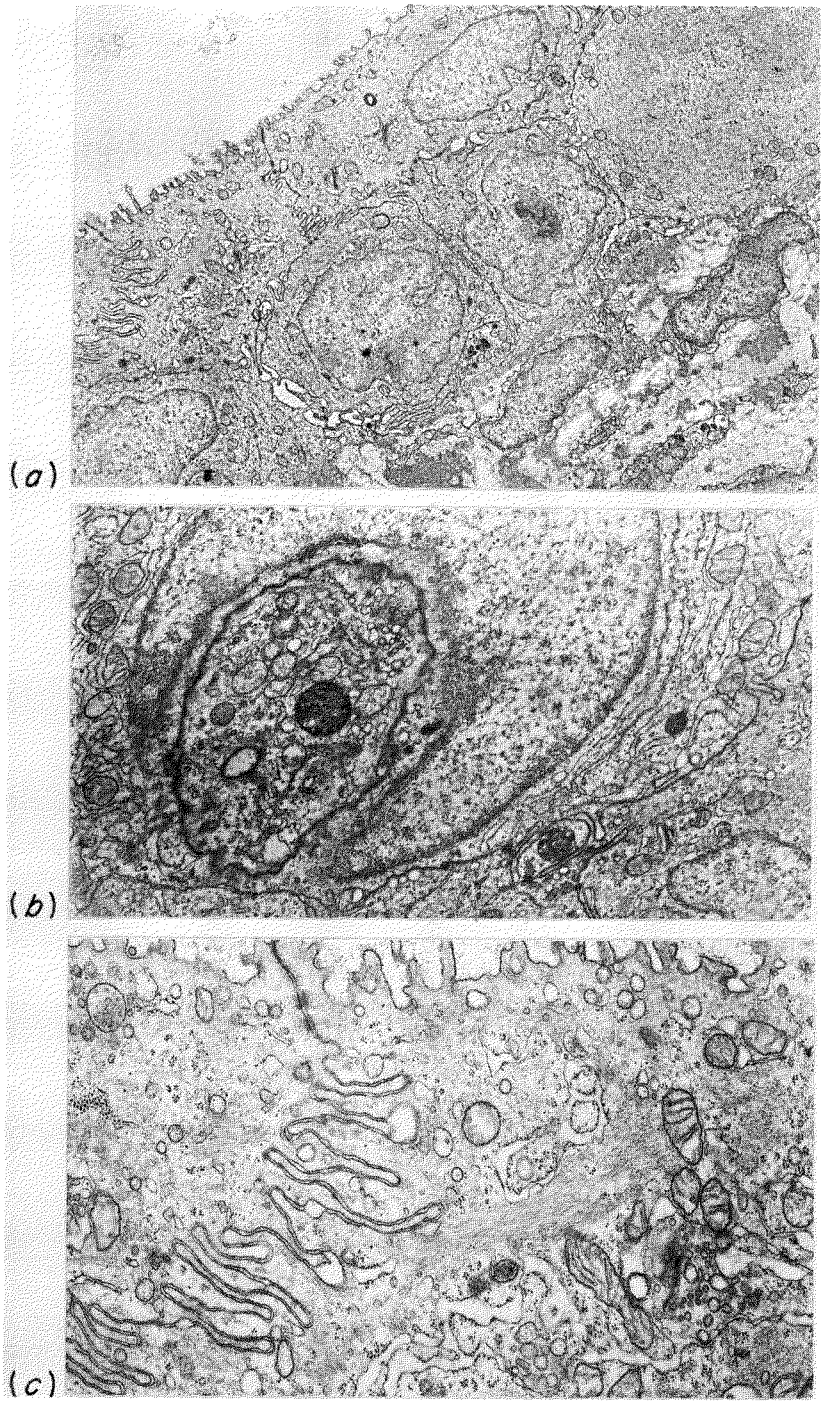
(a)
(b)
(c)

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Fig 2 – Electron micrographs of suckling rat trachea treated with (a) BaP, 10 0 μg/ml 11-day culture This abnormal epithelium is composed of pleomorphic cells without evidence of functional differentiation (mucous inclusions, cilia) 5000X (b) BaP 6 0 μg/ml, 11 day culture A considerably enlarged nucleus almost surrounds an enlarged autophagic vacuole containing dense bodies, smooth-surfaced vesicles of endoplasmic reticulum as well as ribosomes, and other membranous components Parallel arrays of endoplasmic reticulum can also be seen in this cell. 9000X [From E. R Dirksen and T T. Crocker, *Cancer Res*, 28(1968) 906–923.] (c) As for Fig 2a Notice the highly complex cellular inter digitations and the loss of epithelial differentiation. Large amounts of cytoplasmic filaments are seen in the cytoplasm 16,000X

BaP therefore produced morphologic alterations toward abnormally differentiated epithelial states in fetal or suckling members of three orders of mammals in organ culture One question under test was whether this finding indicated probable similarity in the response of living animals from these same orders Dogs and hamsters do develop bronchogenic carcinoma upon exposure to polycyclic aromatic hydrocarbons, while monkeys have not been tested It is reasonable to conclude that target tissues of these orders of mammals undergo biologic alterations upon exposure to comparable concentrations of this polycyclic hydrocarbon

A second question is whether these epithelial changes are pertinent to neoplasia In approaching this question several properties of the organ culture system need to be recognized The concentrations of BaP in media are high, but duration of maintenance is only 3 to 4 weeks BaP does reach epithelium, as determined by autoradiography after incubation with tritiated benzo[*a*]pyrene, but the actual delivered dose per cell is not known This is also unknown for *in vivo* systems using BaP

Invasive neoplasms have not appeared in explants during maintenance Explants treated with BaP, then grafted, have not produced tumors in host animals This may be due to limitations of techniques in grafting, to short duration of contact with carcinogen in culture, or to short duration of maintenance in the immunologically unmonitored culture system before grafting These present defects in the organ culture approach are recognized, but further study of the organ culture system may overcome the foregoing limitations

More persuasive reasons for using the organ culture system are to test metabolic interactions between tissues and carcinogens and to explore inhibitors of the effects of potential carcinogens that man cannot avoid inhaling The hamster data offered at this meeting by Saffiotti *et al* [pp 27–54] and by Montesano *et al* [pp 353–371] contain both causal and potentially preventive approaches to lung carcinogenesis by BaP These approaches are concerned with a carcinogen-vitamin A interaction at the level of the whole animal

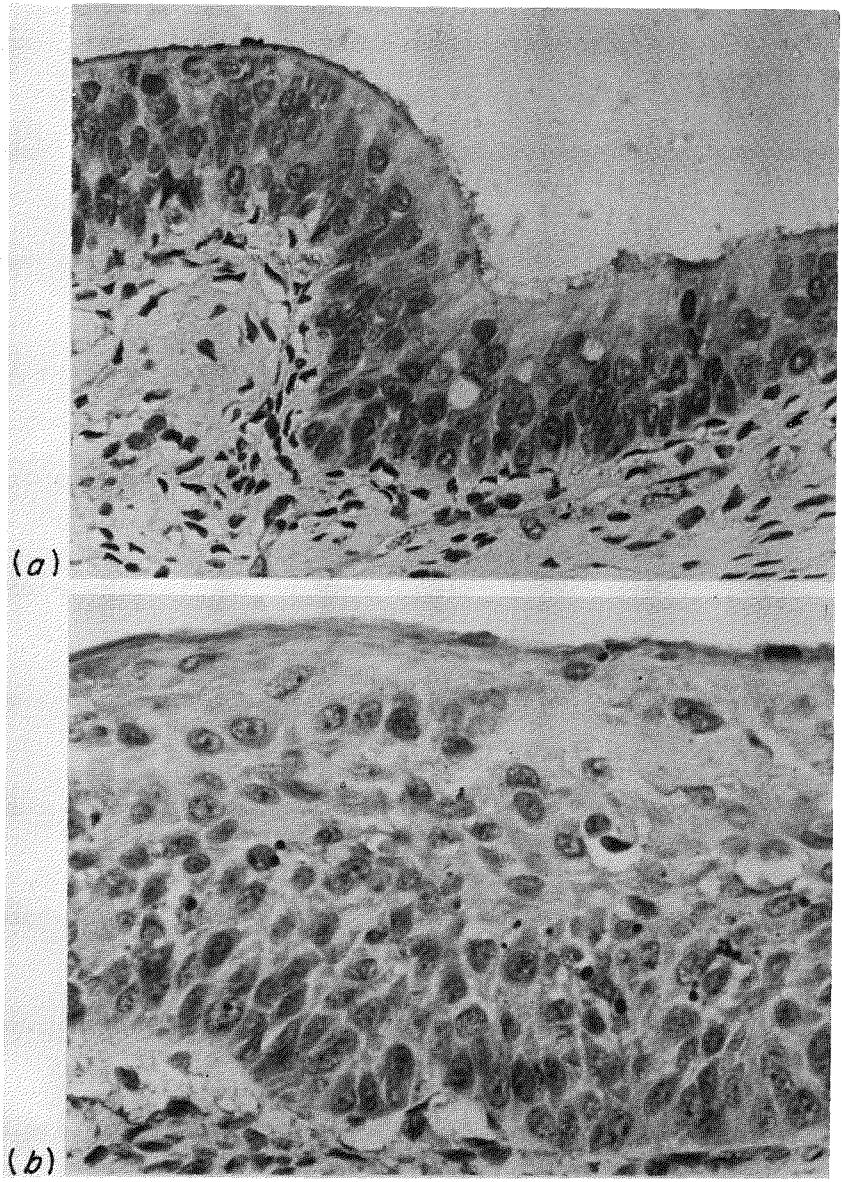


Fig. 3 – Tracheal epithelia of fetal dog. (a) Maintained for 17 days in medium containing acetone vehicle. Pseudostratified differentiated columnar epithelium and cellular connective tissue are preserved. 400X. (b) Maintained for 17 days in medium containing benzo[*a*]pyrene, 15 μg/ml. A high squamous metaplasia with some pleomorphism has been produced. 400X.

The organ culture system permits tests of this inhibitary interaction at the level of the target tissue. The vitamin A effect upon lesions produced by BaP has now been demonstrated both *in vivo* and in culture. Further studies of mechanism of action of inhalant carcinogens and of protective materials are possible in organ culture under controlled conditions of dose and duration of exposure. Finally, since human tissue can be maintained under the same circumstances, protection of human tissue against lesions produced by carcinogens can be tested directly.

DISCUSSION

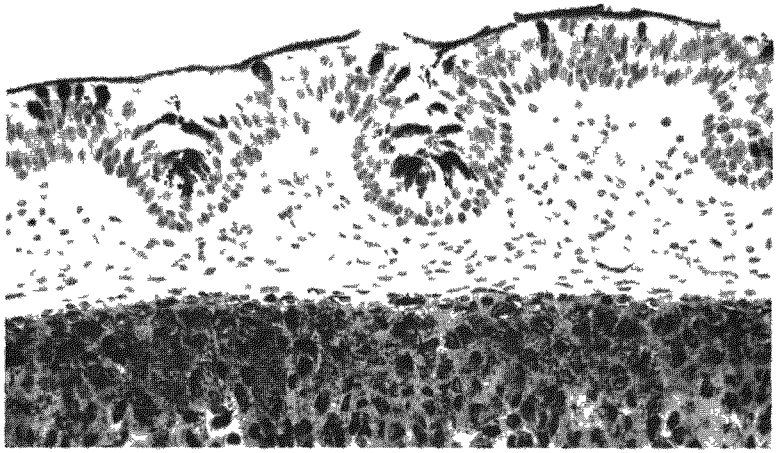
M. G. Hanna, Jr.: I would like to add a comment to Dr. Crocker's presentation. We have no good biochemical or biophysical parameters for measuring the early neoplastic changes in the lung that result from exposure to various chemical carcinogens. Consequently, we have to rely on the assay of the development and growth of lung tumors, which we know can be very markedly influenced by other systems. An example of this, I think, is the possibility of enhanced immune surveillance, which could possibly be interpreted to adversly affect the development and manifestation of lung tumors. I personally relate elevated immune surveillance to the chronic lung changes resulting from the PR8 influenza virus in our experiments [see these *Proceedings,* pp. 305–320], and suggest this as being one of the reasons why influenza may have reduced our spontaneous tumors, and possibly our carcinogen-induced tumors. Too, most of the carcinogens that we are using have also been demonstrated to be immune suppressants. It has been well documented that immune suppression or immunologic inadequacy can influence the number of tumors and the time the tumors can be detected in the animal.

So I think that these considerations, as well as the consideration of the microflora and adventitious infections in animals, must be evaluated. We have seen evidence that adventitious respiratory infections have markedly altered the histopathology one sees during the course of these chronic inhalation studies.

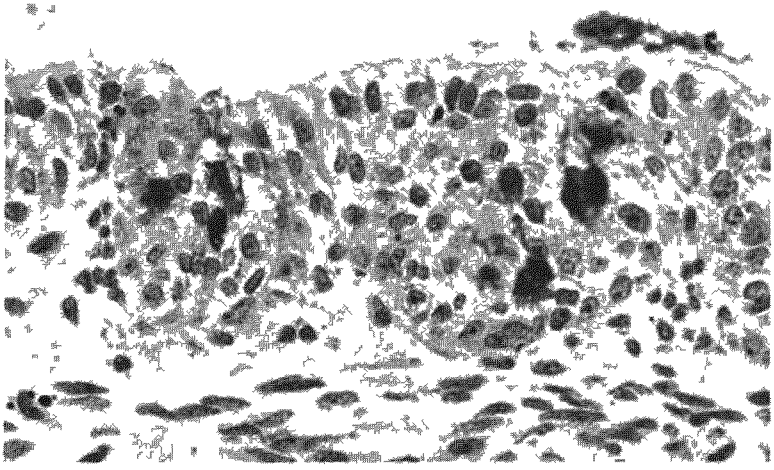
Dr. Roe has recently presented some data showing that germfree mice, which lack conventional flora, also lack the ability to metabolize the carcinogen he was using. Consequently, this factor very markedly affected the number of hepatomas that he achieved in these animals.

Thus, these various considerations, together with the comments that Dr. Crocker has made, may possibly help us develop a model system that we could use for inhalation carcinogenesis.

C. Kensler: I think it was about 12 years ago that Dr. Ilse Lasnitzki of the Strangeways Research Laboratory, Cambridge, England, demonstrated an antagonism between vitamin A and benzpyrene on embryonic mouse prostate, or some other tissue explant culture, and possibly on human fetal lung tissue. At that time we were doing some experiments with painted mouse skins, trying to



(a)



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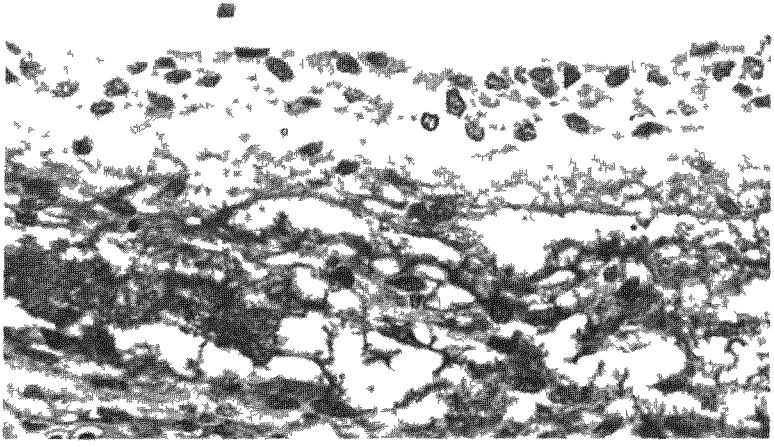

(c)

Fig. 4 – Fetal monkey tracheobronchial epithelia. (a) Tracheal plaque of rhesus monkey maintained for 15 days in medium containing DMSO vehicle. Highly pseudostratified differentiated columnar epithelium is present over cellular connective tissues and cartilage. 175X. (b) Bronchi of *M. cyromolgus* maintained for 11 days on medium containing benzo[*a*]pyrene, 10 μg/ml, in acetone. Epithelium is poorly differentiated, with irregular basal cells, perhaps due to folding or crowding. 400X. (c) Tracheal plaque of rhesus monkey maintained for 8 days on medium containing benzo[*a*]pyrene, 15 μg/ml, in acetone. Connective tissue cellularity is sharply reduced and the undifferentiated pleomorphic epithelium has low levels of DNA synthesis. At 15 days a similar appearance of the epithelium persisted but DNA synthesis was eliminated. 400X.

block the acting benzpyrene with vitamin A. We found in the skin of the mouse that vitamin A applied topically by itself produced an effect on sebaceous glands and epidermal thickening. In view of the hyperplasia produced in mouse skin, are there any levels of vitamin A which produced a hyperplastic response in your culture system?

T. Crocker: Dr. Lasnitzki has certainly done pioneer work in the application of the organ culture system to chemical carcinogenesis. She used vitamin A in association with methylcholanthrene on mouse prostate and applied benzpyrene alone, or combined with cigarette smoke condensate, to human fetal lung. Hyperplastic and metaplastic changes occurred in bronchi of the developing embryonic lung exposed to the latter materials. Dr. Lasnitzki did not study interaction between benzpyrene and vitamin A.

There are no concentrations of vitamin A at which we have found abnormalities of respiratory epithelium. Vitamin A lyses cartilage matrix *in vitro* and *in vivo*. This effect has been extensively studied by Dame Honor Fell of Strangeways Research Laboratory.

The findings you report for hyperplasia of skin produced by vitamin A have also been reported by others and reflect the action of local high concentrations of vitamin A on normally squamous epithelium.

U. Saffiotti: I would like to make two comments. About the vitamin A situation, I think we should pay attention to the site of origin of the lesions under study. The effect of vitamin A on epithelia that are normally squamous, such as the skin or the hamster cheek-pouch, is a different one from that exerted on tissues that normally synthesize mucoproteins. In the latter, vitamin A deficiency switches the cells from the synthesis of mucoproteins to the synthesis of keratin, and its excess protects from the induction of keratinization – and of squamous tumors. In the peculiar tissues where keratin is already normally produced, the mechanism acts on a somewhat different system. Therefore, all the experimental conditions have to be looked at in more detail for a satisfactory interpretation.

My other point relates to Dr. Crocker's report on the possibility of observing further development of the lesions induced in organ cultures of respiratory tract segments when the tissues have been reimplanted *in vivo*. I was looking forward to having Dr. Crocker and Dr. Dontenwill in the same room, because I've always been impressed by a report that Dr. Dontenwill published a few years ago, where tracheas of young hamsters were subcutaneously implanted in the spleens of host animals which were then treated subcutaneously with diethylnitrosamine. The recipient animal therefore received the DEN and carried its own normal trachea in place, plus an additional trachea implanted in the spleen. Papillomas were induced in both tracheas, which meant that the tracheal tissue was the specific target for this carcinogen, and showed that its effect on the respiratory tract was not one to the anatomical location of the trachea, where it can be affected by the exhaled air.

Now this result also indicates that the tracheal segments can be implanted in the spleen and kept there long enough to develop tumors under proper treatment. I've always wondered whether one couldn't combine this procedure with the previous exposure in an organ-culture, and then use the technique that Dr. Dontenwill developed for implantation into the spleen combined with long-term observation of tumor development. The hamster has a very thin, small spleen, and I would like to ask Dr. Dontenwill to give us some details on the technique he used to implant the segments of the trachea into the spleen.

W. Dontenwill: This technique is not very difficult. It's the same one used for implantation into the testes and the ovaries – it's a very simple technique. First, you cut a small section out of the trachea, and then, with a very fine scalpel, you put this piece of trachea into the spleen. It is a little difficult to pull out the scalpel – you must pull very slowly, but the trouble is worthwhile because the reaction is much easier to find in the spleen than in the subcutaneous tissue. Out of about 100 animals injected subcutaneously, only five showed a reaction.

Crocker: I have long admired Dr. Dontenwill's experiment. I have recognized the value in doing what Dr. Saffiotti has just spoken of. We have used diethylnitrosamine in organ culture to determine whether the target tissue can respond, presumably by metabolic conversion of the DEN to an active form. Epithelial lesions are produced in organ cultures of hamster trachea. The next experiment, of course, will be to initiate such lesions in tracheal epithelium in organ culture, and then to graft the explant.

R. Montesano: I would like to mention some work we are doing with Professor Magee at the Courtould Institute of Biochemistry, London, on the capacity of various organs of different animal species to metabolize nitrosamines. We studied the capacity of the different segments of the respiratory tract to metabolize DEN and DMN after exposure of the tissue to labeled DEN and DMN in a conventional Warburg respirometer. So far we have some data for the

trachea and lung of rats exposed to DMN which show the capacity of this organ to metabolize this nitrosamine.

It would be interesting to see the capacity of the respiratory tract of hamsters to metabolize DEN and DMN, because of the different carcinogeneic response of this species to these two nitrosamines. In addition, the level of alkylation of the nucleic acids should allow us to better understand their carcinogenic mechanisms. We would like to do this not only from a qualitative point of view, but also from a quantitative point of view, in relation to the different carcinogenic responses of the various segments of the respiratory tract. The same approach has been used with human tussue, specifically the liver. DMN is metabolized by this organ and shows alkylation of the nucleic acids. We hope that we can use the same approach with other human tissue, such as the lung, in order to obtain a better correlation between experimental work and the human situation.

Crocker: Those are very interesting experiments. Dimethylnitrosamine and nitrosomethylurea were not active in hamster trachea in the organ culture system. We presume that we are encountering evidence that DMN may not be converted to an active metabolite, while NMU may be so active as to react with the components of the medium before effective delivery to the tissue.

STUDIES IN INHALATION CARCINOGENESIS: A CRITIQUE

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ABSTRACT

Current and future work in inhalation carcinogenesis, where studies are made with multiple treatments, would profit from a more conscientious use of factorial experiments. This would enable investigators to examine simultaneously the effects of several treatments, and to determine in advance of an experiment, or a series of experiments, how many animals will be needed to produce results that accurately approximate the "true" condition. Researchers might also consider projects that produce near-certain results with smaller numbers of animals, over a shorter time span. Difficulties with budget changes, high staff turnover, and other problems often lead to faulty results in long-term experiments involving large numbers of animals.

Tomorrow morning, at the last session of the conference, representatives of three federal agencies will tell us about their current and future work in inhalation carcinogenesis. I have something to say about future studies as well as current studies, and all of what I have to say is based on post-mortem observations I have made on past studies.

For about 40 years statisticians have referred to studies with multiple treatments as "factorial experiments." This type of experimental design is a very powerful and useful tool, and all of us would do well to learn more about it than we now know.

The factorial experiment was developed originally to help agronomists study the effects of combinations of different chemical fertilizers on the yield of field crops. Analogy to laboratory experiments in which animals are subjected to a combination of different types of stimuli should be immediate.

For example, let's assume that you are interested in measuring the effect that smog and virus have, individually and in combination, on tumor induction among male and female mice of a certain strain. The number of factors are three

TABLE 1

2^3 Factorial Design, Eight Treatment Combinations

Group	Sex	Virus	Smog
1	M	+	+
2	M	+	–
3	M	–	+
4	M	–	–
5	F	+	+
6	F	+	–
7	F	–	+
8	F	–	–

+, presence.
–, absence.

(smog, virus, sex), and each occurs at two levels (the two levels of smog and virus are presence and absence, and the two levels of sex are male and female). The factorial design, in this case, calls for an examination of 2^3 or 8 groups, as displayed in Table 1, where + represents presence, and – absence.

A complete study would require at least eight chambers, with one treatment combination placed in each chamber. Each replication of this experiment would require an additional eight chambers. When the experiment is completed, the following effects would be available for examination:

1. Sex: (1 + 2 + 3 + 4) *vs.* (5 + 6 + 7 + 8)
2. Virus: (1 + 2 + 5 + 6) *vs.* (3 + 4 + 7 + 8)
3. Smog: (1 + 3 + 5 + 7) *vs.* (2 + 4 + 6 + 8)
4. Sex X Virus Interaction: (1 + 2 + 7 + 8) *vs.* (3 + 4 + 5 + 6)
5. Sex X Smog Interaction: (1 + 3 + 6 + 8) *vs.* (2 + 4 + 5 + 7)
6. Virus X Smog Interaction: (1 + 4 + 5 + 8) *vs.* (2 + 3 + 6 + 7)
7. Sex X Virus X Smog Interaction: (1 + 4 + 6 + 7) *vs.* (2 + 3 + 5 + 8)

I wish to emphasize strongly that all of these "effects" are available for examination from the data of a single experiment. The replications that I alluded to might be introduced to measure variability among chambers, or among laboratories, or among seasons or times of year. But even in the absence of such biases, replications of each of the treatment combinations might be necessary to provide an adequate number of animals for valid inferences about the results of the experiment.

No matter how elegantly an experiment of this type is designed, it will fail in its mission if an insufficient number of observations is taken. I will clarify this statement with the following example. If you tossed a coin ten times and observed seven heads and three tails, would you say the coin is biased? No! Take the same coin, toss it 1000 times, and observe 700 heads and 300 tails. Would

you now say the coin is biased? Yes! What has happened to make you change your mind? You might say, "I have more faith in the second experiment because it is based on many more observations," and your intuition would be perfect. Why not apply the same intuition to experimental situations? For example, why is it so difficult to accept the fact that you may bet 9 to 1 but not 19 to 1 that a 2% incidence in lung tumors among 500 animals in one experimental group is different from a 4% incidence of the same lesion in a second group of 500 animals – that a similar difference based on 1000 animals in each group would allow you to bet 19 to 1?

The determination of adequate sample sizes can and should take place prior to the performance of an experiment. What has been lacking in some of the research we have been doing is intellectual honesty. If you propose to examine the effects of certain stimuli because you feel that these stimuli will produce a doubling in response, then why not make an observed doubling more believable by taking enough observations to allow you to back it up with a 95% probability statement, or with 19 to 1 odds? The decision to do this can be made prior to the experiment.

Don't be upset with the statistician who tells you that the apparent doubling effect which you observe is not significant at the 95% level. All he is telling you is that the same doubling would be more believable if it were based on more observations.

An example of the role that prior planning can play in determining adequate sample sizes is given in Fig. 1. The ordinate scale represents an observed n-fold difference between two groups, and the abscissa displays the percentage of animals with tumors in the entire experiment. The curves labeled "135 (and 270) animals per treatment" are such that *observed differences* above the lines will be declared significant with 95% confidence.

In a 2^3 factorial experiment, with 135 animals per treatment combination, 1080 animals are available for observation. The main effects and interactions, which I mentioned earlier, are estimates based on the difference of two quantities each of which is a summary of the observations on 540 animals. Which of the two sets of 540 animals is used depends on the particular effect being considered. The important thing to realize is that the same 1080 animals are being used to measure and test all of the effects.

Let's say that we expect to see lung tumors in 3% of all the animals in this experiment. If, when the experiment is completed, we observe a lung tumor rate of 2% in the 540 animals living without smog, and a 4% rate in the 540 animals living with smog, we will not be able to say with 95% confidence that this doubling is significant.

In fact, we know before we start that, with 135 animals per group, we could not declare as significant (at the 5% level) an observed tripling effect (*i.e.*, a 1.5% rate in non-smog *vs.* a 4.5% rate in smog).

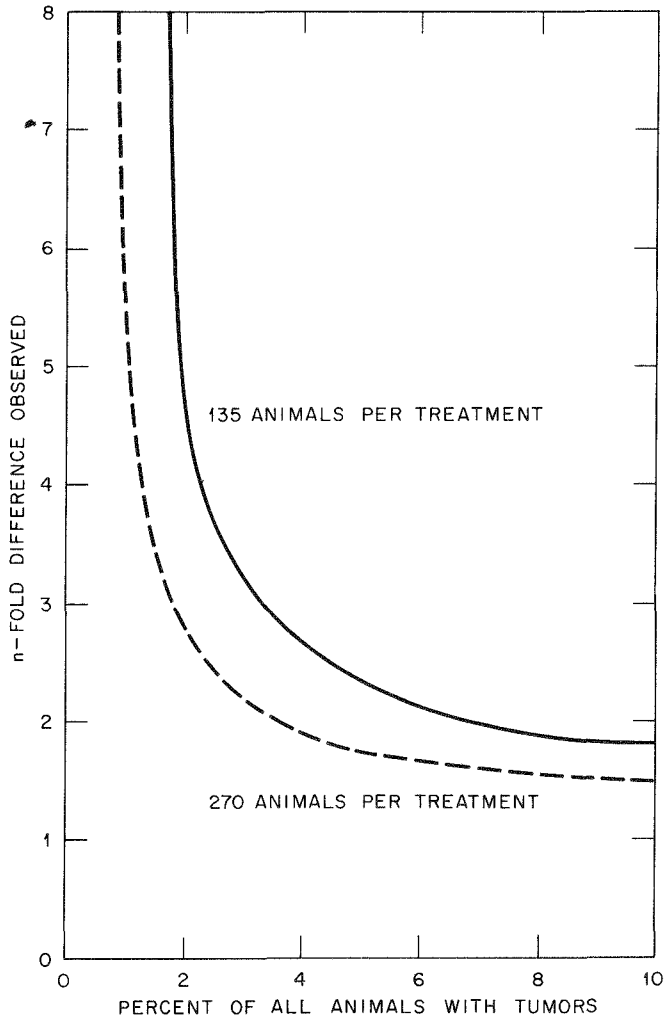

Fig. 1 – Sample-size requirement for a 2^3 factorial design. Observed n-fold differences above the line will be declared significant (at the 5% level).

By the same token, if we expect to see a tumor rate of 3.5% in an experiment involving 2160 animals (270 per group), then we will be able to declare an observed doubling as significant, with 95% confidence.

In concluding I would like to make a further plea for better and more honest planning, especially as it pertains to long-term animal experiments. Experience has led me to conclude that such experiments are hopelessly cumbersome and exceedingly wasteful. They are not optimal in any sense – least of all in the sense of achieving maximum information for minimum costs. Their original purposes, which may never have been clearly defined at the outset, are generally

lost sight of long before the experiment ends. Where protocols might have existed, they have been abandoned somewhere along the line. The requirements for sustained support of large and competent staffs are minimized from the outset. As a result, continual turnover of personnel is responsible for horrible biases in animal care and observations, and in data collection and record keeping. These situations have persisted, do persist, and will continue to persist where large, long-range experiments are being performed.

My plea, therefore, is that we plan intelligently and honestly, and in our planning give serious consideration to small, short-term, single-purpose experiments which are likely to give meaningful results. It occurs to me that, as responsible scientists, we are interested in conclusions and decisions, not in warehouses full of data or in fat summary reports which say nothing and mean less.

ACKNOWLEDGEMENTS

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DISCUSSION

Y. Alarie: This paper is very good! It should have been placed at the beginning of the conference.

I would like to give an example that should emphasize one of your statements. We have an exposure chamber in which we can place 100 rats, and usually instead of putting 100 male rats and leaving it at that, we place male and female such that we now have groups of 50 and 50. Then we want to know about a pretreatment, one in the male and one in the female, so now we have groups of 25 animals. What we end up with are small groups of insignificant size.

You should be congratulated for making these comments in such good terms.

M. Kastenbaum: I have one comment on what you said. The statistician generally comes into the picture at the tail end of experiments, so I'm accustomed to being right here at the end.

N. Nelson: Well, this is not quite the end yet, you know!

Kastenbaum: That's right. There is tomorrow, and the cocktail party does come tonight!

U. Saffiotti: I want to express my very strong congratulations for your very well-presented thoughts. I subscribe to them, and I certainly want to have as much of this type of input in the future planning of our activities in the field. This is where the situation begins to get more complicated: How do we get the biologist, who is planning his experiment, to benefit from this kind of background, and calculations, in advance?

There are not very many biometricians who have been acquainted with the problems in this field of experimental study. The critical evaluation of such programs requires consideration of the design of the experiment in order to meet the various biological requirements of the study, such as variation in mortality and multiple effects that may interfere with each other.

What I think we should really try very strongly to obtain from you and your colleagues in the very near future are very precise guidelines. These should be readily available to the investigators, not *a posteriori* but *a priori,* and not just in general terms but in the form of manuals. These should be systematic listings which include statements of significance for certain types of incidences and evaluations of numbers of animals that would be required for the design of the experiment.

I am not going to leave it at that, and I hope in the next few months we can establish a better contact. This is the kind of association that has to be developed and available in the planning of future experiments.

M. G. Hanna, Jr.: I would like to add another comment; that is, I recommend that all investigators designing long-term inhalation studies make friends with their neighborhood statisticians and take time to get acquainted. What we found it takes is communication between the biologist and the mathematician. A mutual education between the two is essential so that the biometrician is aware of the biological problems and the biologist recognizes the statistical limits.

F. McIver: I would like to also commend the attitude of the talk, but I would like to seriously point out that if you really knew what fold effect to expect, you really wouldn't need to do the experiment.

Kastenbaum: I would like to make a comment on that, Dr. McIver, at the expense of being facetious. When I spoke of intellectual honesty, I was addressing myself to that point. If you embark on an experiment because you expect deep down to see a 20% incidence of lung tumor, and, at the end of your experiment, you observe only a 5% incidence, don't feel that you can make the same strong inferences about the 5% as you might have made about the 20%. Be happy with what you get!

A. P. Wehner: I don't think anybody can argue with the points which were brought forward so clearly. However, I would like to submit for consideration that it is not always the ignorance of the biologists, but sometimes the available means, which set limitations to the experiments.

W. Dontenwill: I have one comment. In our experiments with mice we had 225 mice in each group. We entered these 225 animals into the experiment in five series over a period of time. The results of this study suggested that there was an interaction of the effects with time. Would you comment on this point?

Kastenbaum: Replication in time has always been a serious problem. One of the things that you need to remember is that the only reason that you are performing experiments is so that you can make some inferences from the particular to the general. If you perform experiments in time and you observe a variation with time, you should not average over this variation. If you do, you are just deluding yourself into thinking that things are less variable than they really are.

If you need to replicate in time, and there is a time effect, then this effect may interact with all the other effects in the experiment. Under these circumstances you should exercise extreme caution in the inferences you draw.

Saffiotti: I want to speak for a moment on the point that Dr. Wehner has raised, of having to balance the amount of effort and available resources one can put into a particular experiment. I do not think that limitations in resources or budgets are a justification for doing an experiment badly. I think that the obvious guideline is that if something is worth doing, it is worth doing well. To establish an expensive and complicated experiment without any previous statistical evaluation may seriously limit the ultimate analysis of the data.

Kastenbaum: I'd like to make one further comment on that point. What I presented in my talk were 95% confidence statements. This 95% probability is a figure that we see very often in all scientific literature. Somehow we associate an aura of sanctity to an event if we are willing to bet 19 to 1 on it. But I ask you to search your souls and tell me how many times you are really willing to bet $19.00 to $1.00 that the statements you make are correct. Shouldn't you rather be betting 3 to 2, or 6 to 5? The point that I really want to get across is that you can start out in your experimentation in this way. If you can't afford 200 animals, but you can afford 100 animals, why not start out by saying, "I'll be satisfied with a 9 to 1 bet rather than a 19 to 1 bet."? Are you willing to do this? You can make this decision before you start your experiment.

F. G. Bock: Can one plan to set up a two-stage experiment where the first stage is a screen for the second stage?

Kastenbaum: Yes, it can be done, and has been done – and done honestly. An example of this is the study of somatic effects of low-level radiation at Oak Ridge. The sample size prescribed indicated that if, at the conclusion of this experiment, we observed a significant effect, we were home free. If not, then we could not say that no significant effect existed. Instead, we would have to continue with the second stage of the experiment. You, too, can embark on your research this way, knowing full well what the implications are.

D. Hoffmann: Years ago, some form of lesion, presumably tumors, was observed in two rats. This effect was associated with the components of an insecticide. Although this observation was not significant on a statistical basis,

the decision was made to remove the particular insecticide from the market. As it turned out, it was good judgment. Now where did statistics bring us in this case?

Kastenbaum: I will make a philosophic statement in response to your question. There are certain inferences which we make on the basis of an assumed knowledge of the true probability structure underlying the outcome of an event. There are also such things as degrees of belief and subjective probabilities. These latter phenomena come about as a result of things we see or think we see. They border on feelings which may arise from circumstantial evidence. When you contemplate an experiment, you are dealing on this level. You hope that the results of your experiment will add weight to your evidence.

I am never in a position of proposing that a particular biological experiment be done. Most of you are. The evidence you have presented at this meeting and the future experiments which you propose are designed to bolster, or shatter, the notions which some investigators have concerning the induction of malignancies. Ideas and notions may be based on as little as two, or even zero, observations. Proofs require much more work.

Nelson: Before I close the discussion, I would like to call on the distinguished Dr. Alexander Hollaender, who has several comments on the genetic aspect of carcinogenesis.

A. Hollaender: During the discussions of the last few days I have hardly heard the fact mentioned that some carcinogens can break chromosomes, and often produce gene mutations. While many interesting things have been brought out, the genetic effect of carcinogens has not been discussed. Not all carcinogens are mutagenic, but many are, and many mutagenic chemicals are carcinogenic. It is sometimes technically difficult to detect mutagenic and carcinogenic effects, but there is a definite relation between mutagenesis and carcinogenesis. We do not know the dimensions of this relationship; in fact we know very little about it. I think any study of carcinogens should include their effects on chromosome aberrations as well as mutation production.

There are some excellent reviews available in this area. An especially good one that has just come out is the "Report of the Secretary's Commission on Pesticides and their Relationship to Environmental Health," Parts I and II, U.S. Department of Health, Education and Welfare. This is a concise review, but enough details and references are given to make it most useful. On pages 565–677 there is a good list of methods now available for detecting chemical mutagens. A book sponsored by the Environmental Mutagen Society on Methods for Detecting Chemical Mutagens will be published by Plenum Press in the near future. This volume will give a more detailed discussion of different methods available for the detection of chemical mutagens.

There is no single, all-inclusive method to obtain a comprehensive picture of the effects of chemicals on mutagenesis. It is necessary to use four or five

methods to be fairly certain that all chromosome aberrations and mutations produced by the chemical are detected. It is probably from studies such as these that considerable information will become available, and I believe these approaches should be included in future discussions of carcinogenesis.

Some of the methods I described use microorganisms which give a quick answer in regard to mutagenesis. Final checks should usually be done by host-mediated and dominant-lethal study. These are all described in the references I have given. Chromosome aberration studies are being done extensively in medical schools and hospitals – places where these techniques can be learned – and they should prove very useful in connection with carcinogenesis studies.

This has been an extremely interesting meeting from many aspects. However, I am sorry this area of genetics was not included, and I hope that future conferences on carcinogenesis will take this important area into consideration.

ACTUARIAL METHODS IN THE EVALUATION OF DATA FROM LONG-TERM ANIMAL EXPERIMENTS

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A distinction has to be made between qualitative and quantitative experiments. An extreme example of the former would be if, after the administration of a new drug, a mouse stood on its head for 5 minutes. It might be wise to repeat such an experiment, but insofar as the knowledge and experience of the investigator permitted him to regard the observation as "unique" and a form of behavior that has not previously been recorded, formal statistical evaluation would be superfluous.

Perhaps a more serious matter is the inappropriate use of statistics in experiments in which animals have not been adequately randomized between treatment and control groups initially. Proper randomization of animals is a prerequisite for a quantitative experiment in which statistical evaluation is contemplated.

Equally important, particularly in the case of life-span studies, is that animals should be observed on every day of the week including Saturday and Sunday. Postmortem autolysis occurs rapidly in mice and, in all species, detailed histopathological evaluation is rendered increasingly difficult as the interval between death and necropsy increases. In long-term mouse studies, a few mice (up to 5%) are usually "lost" for the purpose of pathological evaluation despite the utmost care and 7-day per week observation, but for every day of the week that the laboratory is closed, a further 15% of the animals are likely to be rendered unavailable for pathological study. It is common to see salvage rates of the order of only 66% for mouse experiments conducted in laboratories which are closed on Saturdays and Sundays. Whatever statistical analysis is applied to the results of such experiments, the answer obtained may be misleading.

In our laboratory we kill sick animals, rather than wait for them to die, because we believe that it is more important to be able to make a sound pathological evaluation than to avoid the possibility that, by killing a sick animal that might have recovered, one would bias the results of an experiment. With

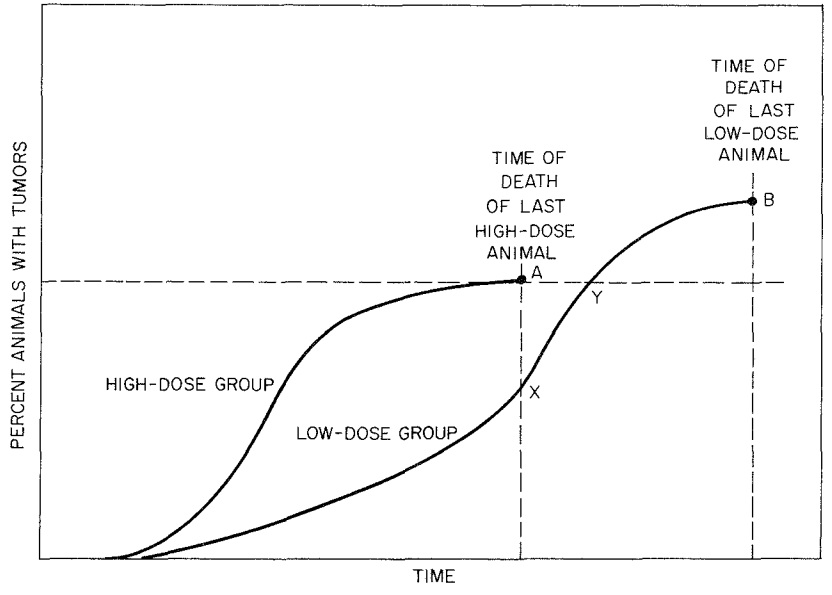


Fig. 1 – Commonly used ways of expressing data from carcinogenicity experiments.

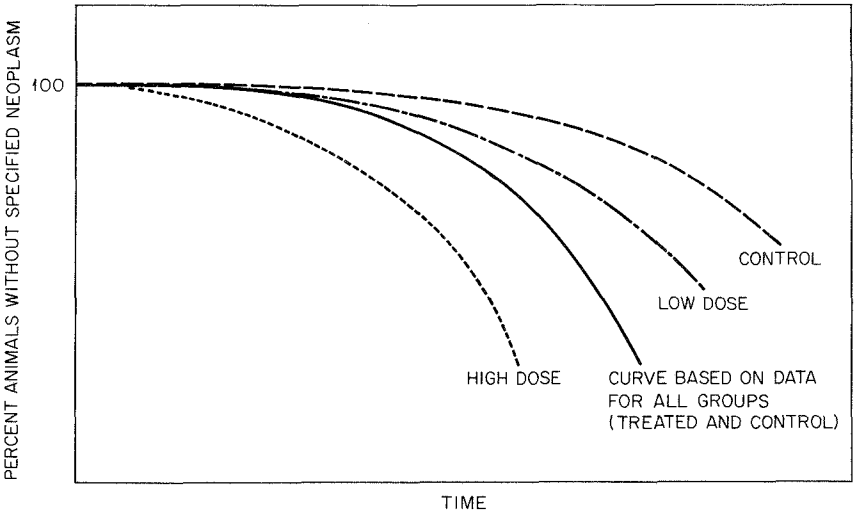


Fig. 2 – Calculation of risk of development of specified neoplasm in differently treated groups. The overall experience of each group can be described quantitatively and, if required, significance levels can be calculated in respect of apparent differences.

deliberately killed animals, we are able to follow a regular necropsy regime. This would not necessarily be possible if animals were permitted to die.

At this conference we have heard speakers refer to "tumor incidence" in response to different forms of treatment, and frequently such reference has been made without mention of comparative survival times. Commonly used ways of expressing data from carcinogenicity experiments are illustrated in Fig. 1. If a test material is toxic it is possible for the final percentage of a group that developed neoplasms to be higher in a low-dose group (B) than in a high-dose group (A). Without qualification with regard to survival, this result would be misleading to say the least. Comparison of percentages of tumor-bearing animals in the two groups at a stated time (*e.g.*, A and X), or of times by which a given percentage of animals in a group have become tumor bearers (A and Y) could also be misleading, and I make a plea for the use of actuarial methods in the evaluation of data from laboratory animal experiments.[1–4] A continuous adjustment for intercurrent (or nonrelevant, *i.e.*, nontumor) deaths is made, and a tumor-free survival curve is constructed which takes the form illustrated in Fig. 2. This curve shows the estimated survival of the animals if tumor development were the only cause of death. The expected shape of a curve for a group of animals depends only on the tumor incidence rates at various ages and not at all on the death rates from other causes, such as toxicity on local epidemic infections. The curves for the individual groups can be compared numerically by the method of Gehan[5]. Each curve is built up on the basis of numbers of animals developing tumors as proportions of animals alive and at risk of doing so at serial points in time. In other words, each curve is an expression of "time-standardized risk." The burden of my argument is simply that we should abandon the concept of percent of animals that develop neoplasms in favor of the concept of "time-standardized risk."

By the use of this method of analysis we are currently obtaining far more information from long-term studies than was previously possible. Sometimes actuarial analysis shows that a conclusion based on crude analysis was completely wrong.

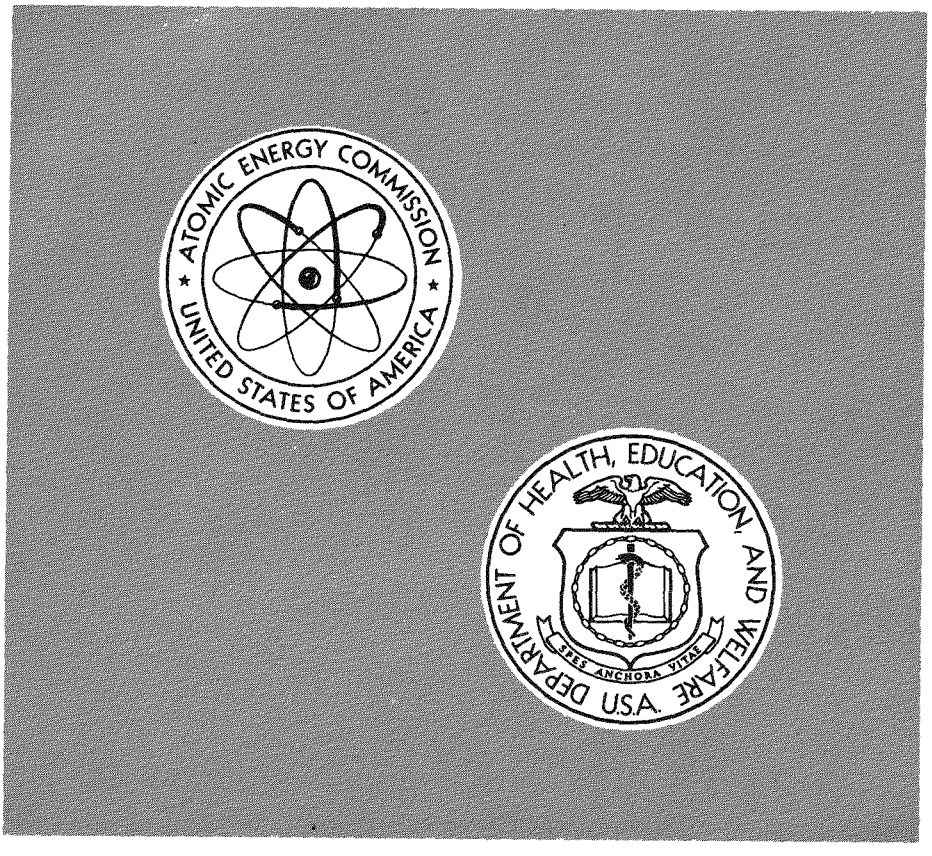
REFERENCES

1. J. O. Irwin and N. Goodman, Statistical treatment of measurements of carcinogenic properties of tar and mineral oils. *J. Hyg.*, 44(1946) 362.
2. C. C. Spicer, Estimation of tumour susceptibility in pure lines. *Brit. J. Cancer*, 1(1947) 298.
3. H. I. Pilgrim and J. E. Dowd, Correcting for extraneous death in the evaluation of morbidity or mortality from tumour. *Cancer Res.*, 23(1963) 45.
4. M. C. Pike and F. J. C. Roe, An actuarial method of analysis of an experiment in two-stage carcinogenesis. *Brit. J. Cancer*, 17(1963) 605.
5. N. Mantel, Ranking procedures for arbitrarily restricted observation. *Biometrics*, 23(1967) 65.

SESSION V

SUMMARY OF CONFERENCE, AND PROGRAM PLANNING IN INHALATION CARCINOGENESIS BY NCI, USAEC AND NIEHS

Chairman – Carl G. Baker
National Cancer Institute
Bethesda, Maryland



SUMMARY OF CONFERENCE

CARL G. BAKER
National Cancer Institute, Bethesda, Maryland

I will formally open the session by expressing thanks, for all of us here, to the Organizing Committee and the Oak Ridge National Laboratory staff, including the lovely ladies who helped keep this both a productive and a pleasant meeting.

It may be useful to look at the objectives that Dr. Hanna proposed at the beginning of the meeting and ask ourselves how nearly we achieved them. My overall impression is one of a generally fine meeting, with a good review of many topics. Some of the information reviewed here was well known to some people, but not to others in different areas. Thus, *in toto,* I am sure all of us received important information in certain areas unfamiliar to us. Further, the mix of technologists in the inhalation field with biologists in carcinogenesis was a good one. Another good combination resulted from the bringing together of people from various kinds of institutions: universities, research institutes, government agencies, and industries.

Also, I would like to express appreciation that we had with us a number of visitors from across the Atlantic, but I regret the absence of three leaders in the field: Dr. Ernest Wynder, Dr. Paul Kotin, and Dr. Philippe Shubik. We all missed them very much.

Dr. Hanna's first objective was to provide a general evaluation of inhalation technologies for the study of respiratory carcinogenesis; in this objective, I think we succeeded rather well. The review of the state of the art during the symposium on Wednesday, Relation of Inhalation Exposure to Carcinogenesis, plus several other papers throughout the week, succeeded well in supplying this kind of evaluation and review.

Dr. Nelson initiated the symposium with general considerations of the problem in man, identifying a number of hazards and approaches to the problem. Importantly, he reminded us that much of what we are doing is really

to be evaluated in the long run by our success in reducing cancers in man. Dr. Saffiotti stated that every eight and a half minutes someone in this country dies of lung cancer. The figure for cancers in general is one death every two minutes; I'll leave it to you to calculate how many people have died since this program started.

Other papers in the symposium reviewed the state of the art quite succinctly: they covered the chemical nature of hazards inhaled; the factors involved in the study of respiratory carcinogenesis, including equipment, technological procedures, and experimental design; the uranium miner problem; and the associated quantitative studies in animals on lung tumor induction. Dr. Kuschner provided us with sound counsel on the meaning of "lung tumors," reminding us that there are different kinds, and he commented repeatedly and wisely on pathology throughout the meeting.

Finally, in the symposium on the first day, the interesting issue of mathematical models was brought to the fore; as usual, we had vigorous discussions on the values and shortcomings of mathematical models in biomedical research and on whether the model was actually giving us sufficient information about the real-life situation. Dr. Schneiderman suggested that perhaps the apparently divergent opinions on this subject are not so far apart. The problem here is related to the more general one of approaching similar problems from different organismic levels. Because respiratory carcinogenesis can be examined from such different standpoints as populations, individual whole organisms, organ sites, tissues, cells, and subcellular components, the use of different tools for these approaches provides somewhat different conclusions, and this sometimes leads to vigorous arguments. The problems of how one views the issues, selects tools, and approaches and resolves these differences are important and, as regards carcinogenesis, these problems relate to the final session of this meeting.

Even while we remember the treacherous assumptions that often underlie mathematical models, some of us who are dealing with very complicated situations in biology and medicine look perhaps with envy at the beautiful simplicity of some of the mathematical models. When we see the families of curves presented on, say, relationships between the size of aerosol droplets and their aerodynamic properties, I think many of us wish we were able to describe this kind of order in biological studies and show such rich interrelationships. Sometimes such simplicity is possible for biological systems, but too frequently, as in the case of cancer, it is not – at least, not yet.

Much of the problem depends, of course, on the validity of the assumptions used in setting up a model, so this topic is always a lively one. In many instances models, if their assumptions are not too awry, are often very useful in predicting results.

In the area of radiation carcinogenesis, we have been able to proceed a considerable way with the cumulative effects of different forms of radiation, and the concept of body burden has been useful in this respect. Although attempts

to apply this concept in the chemical carcinogenesis area have not been too successful, it could be very useful for practical handling of problems in prevention; hence more thought and effort on the problem are needed.

The first session on Thursday, Inhalation Technology, provided a wealth of information on germane technologies. Many of the reports were of very practical help for those of you who are actually conducting experiments, and I was particularly struck by how the discussion during this period, as well as at other times, generated information that was very useful in answering a number of important questions. This profitable exchange of information was another very valuable outcome of the meeting.

Session III, Respiratory Carcinogenesis, provided some examples of studies on biological response at the cellular and whole organism levels. The idea that injury or damage to tissues preceding exposure of the tissue to carcinogenic agents is a key or necessary element in cancer transformation was suggested during this session and at other times; but I think that even though this was a recurring theme, we did not really clarify the issue appreciably. A future session on this subject alone might be worthy of consideration.

Another important outcome of Session III was discussion of the possibility of easier, short-term *in vitro* assays for carcinogenicity. Here I caution that the validity of these assays in terms of *human* carcinogenesis must be determined before their value can be assessed. This theme of relating animal studies to man is one of our most difficult problems, and though it ran throughout the meeting, it was not always clearly resolved because of its great difficulty. I would like to return to this point later.

On Friday, real progress in respiratory carcinogenesis was demonstrated, and the presentations showed many important quantitative results. As Dr. Saffiotti had reminded us earlier, this field is relatively new, yet in the last three to five years progress has been made in producing tumors of the respiratory tract, including cancers. The associated techniques and developments are extremely important for exploring systematically the various factors involved in respiratory carcinogenesis. From these excellent reports it seems evident that what is necessary in the field of respiratory carcinogenesis are large-scale, multi-disciplinary types of investigation.

The report given by Dr. Montesano demonstrated quite clearly how an agent that itself is not carcinogenic for the lung and that can be administered by a route other than inhalation can influence very significantly the lung tumor incidence when a tumorigenic agent is administered by way of inhalation. This question of multiple exposures, one of our other difficult problems because of the number of variables involved, faces us as another area demanding more attention. These studies were important in clarifying in a quantitative way some of the effects of multiple exposures, even by multiple routes of administration.

Finally, Dr. Kastenbaum forcefully reminded us of the requirements of sound experimental design and of the necessity of using sufficient numbers of animals to permit conclusions based on a probability that most of us would

prefer to have in drawing conclusions. I would like to suggest, also, that the key to the use of computers and to the comparison of results from one group to another, or from one group of experiments to another, lies in this careful attention to experimental design. Too, Dr. Roe reminded us that qualitative experiments or, I would also say, certain types of probing experiments do not require as many animals as those follow-up studies demanding more extensive and careful quantitation. I think both points are very well taken, the choice between them depending upon the purpose of the efforts.

Dr. Hannâ's second objective was to determine: What are the essential problems of respiratory carcinogenesis that can be answered only with inhalation techniques? Here I would conclude that we were less successful than in reaching the first objective; yet this theme permeated the meeting, and many of the studies did allow conclusions that were pertinent to the question. Explicit germane issues came up, for example, in the discussion of Dr. Dontenwill's work on hamster exposure to cigarette smoke. Comparisons were made between results obtained in mouse skin painted with either fractions of smoke or whole smoke condensate and in smoke inhalation in hamsters. However, comparisons of mouse skin response to condensate and hamster respiratory tract response to smoke remained at the descriptive level, and little gain in understanding resulted from this discussion. Certainly many of the reports were directly pertinent to the question, but we did not answer it satisfactorily.

Dr. Hanna's third objective was to learn whether we have a clear understanding of inhalation experiments. If one does not quibble too much about the finer meanings of this question, I think the answer is that we have come a long way toward an affirmative answer in the past three or four years. Friday's reports were particularly important in this regard.

The fourth objective Dr. Hanna presented to the conference was to answer the question: How do inhalation experiments relate as models for human respiratory cancers? Here I would conclude that we did not succeed, but discussion of the topic was frequent and often lively. How we relate animal experiments to the human situation, or (as I prefer to say it) how we assess their significance for man, is a topic deserving much greater attention.

If we ask ourselves how we go about telling whether our experiments done in the laboratory are of valid significance for man, we conclude that somewhere along the way we must tie together and correlate data from the animal experiments with data on man. The bottleneck in doing this, it seems to me, lies primarily in attaining sufficient information about *man,* which raises the question of shortages in epidemiology and biometry and the problem of finding individuals who are willing to tackle difficult and long-term studies in man. Yet without this type of data I do not see how we are going to successfully relate the animal studies to man. So I make a plea here, to those of you in the medical schools, to promote more respect for epidemiology and preventive medicine than I see in medical schools now. We must also devote more attention to the training of epidemiologists; even the kinds of epidemiology that are currently

taught are not often helping us to obtain critical answers in the carcinogenesis field. Again, this is probably because the problems are long and difficult, and they may lead to negative results even after three to five years of effort.

We need to try to find ways to shorten and simplify studies of this type. If cancers were reportable diseases, even if only in industrial settings such as in Great Britain with reports of the Inspector of Factories, then extremely important information would be readily available. Improvements in record linkages suggested by the U.S. National Committee on Vital and Health Statistics (National Center for Health Statistics, Series 4, No. 7, 1968) offer possibilities for obtaining some of these data without the necessity of the long-term, typical epidemiologic studies. It is hoped that these steps will be implemented.

We are currently undertaking the Third National Cancer Survey, which is a follow-up on the 10-city surveys of 20 and 30 years ago. These studies are designed to obtain representative information on incidence figures in cancer and additional information on occupational experiences of the individuals. Data on economic and other factors will be sought in a subsample group. We will be attempting to identify every new cancer case in a three-year period around the decennial census in metropolitan and rural sampling areas, with a total of something like 25 million people. We anticipate about a quarter of a million cancer cases in this period.

By using some of the data available in the biometry and epidemiology branches in the Cancer Institute and by working with the American Cancer Society, we will be able to provide the kind of base line that will allow us to tell you about how many deaths there will be this year, as well as the trends in these deaths – how some cancers, like stomach cancers, are going down, and how lung cancer is going up, and so on. This activity seems to some people rather mundane, a mere collecting of numbers, but I submit that it is a very important function, for it allows us to make significant correlations. I am glad we have people who are willing to tackle these difficult studies. Dr. John Bailar is in charge of this Third National Survey.

Now any meeting, of course, lasts a finite period of time, and it is impossible to cover all the topics one could think of. I would, however, like to mention some important areas not covered so that they may possibly be considered for future meetings. I have already mentioned epidemiology as being a highly important aspect. At the beginning of the meeting Dr. Nelson alluded to the identification of hazards for man, and I hope that further consideration can be given to how that is to be done. My conclusion is that much more *systematic screening* than we have been doing is required. This kind of work, again, is not too popular with many investigators, but if one poses the question: How do we identify agents hazardous for man that are introduced into society every year?, then some *larger scale, systematic* assay effort seems to me to be in order. We have learned a lot in the past, from experiences in the cancer chemotherapy area, on how one does this type of screening on a large scale. Many of the problems

that have been faced and solved in chemotherapy are very important and useful for an expanded effort in chemical carcinogenesis screening.

The areas of chronic toxicology and pharmacology, as far as carcinogenesis goes, seem to me to be deficient. Many departments of pharmacology throughout the country are not interested in this kind of pharmacology, and again the old problem of long-term experimentation and requirements for large-scale animal facilities raises its head. We should give additional attention to aspects of pharmacology and toxicology suitable for dealing with improved assay and mechanism studies in carcinogenesis.

The problems of multiple exposures, with the large numbers of variables that must be considered, raise very difficult problems for those of us interested in chemical carcinogenesis. A future conference on this subject might be in order.

We did not talk much about the role of viruses or of immunology in cancer causation, or about another subject that is always good for long, vigorous arguments – the pathological end points to be used in determining the results of carcinogenesis experiments. These are areas of considerably high importance, but there is not enough time in this session to cover them.

The great amount of talent represented at this meeting and the tremendous amounts of effort represented by the reports given here make me think of the pressing need to bring these talents and efforts together in perhaps a more coordinated fashion. This thought sets the stage for the session to follow, which is concerned with program plans. These will be discussed by a panel covering plans from three government agencies.

PROGRAM PLANNING IN INHALATION CARCINOGENESIS: NATIONAL CANCER INSTITUTE PLANS

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ABSTRACT

A Special Lung Cancer Program has been established by the National Cancer Institute during fiscal year 1969. An appropriation of $1,153,000 has been made for fiscal year 1969 to implement the initial phase of this program through contract-supported collaborative projects. Existing collaborative projects in this field, supported by the Carcinogenesis Area contracts, are being further developed and coordinated with the new efforts. A new Lung Cancer Unit is being established in the intramural laboratories of the Carcinogenesis Area. — Particular emphasis is being given to the problem of tobacco smoke and the development of a less hazardous cigarette, with advice provided by the Tobacco Working Group established by NCI on the recommendation of the Lung Cancer Task Force. A plan has been developed from these recommendations and is articulated in the following phases: selection, preparation, and characterization of different types of tobacco and tobacco smoke condensates for subsequent bioassay and analysis; bioassay of tobacco smoke and its fractions in a battery of animal systems; chemical analysis of tobacco smoke and its fractions; evaluation of the data obtained in relation to the human situation. — Considerable effort is being given to further development, characterization, and definition of biological models appropriate for the bioassay of respiratory carcinogens and for the study of key factors involved in their carcinogenic activity. — In view of the key role of particulate materials in respiratory tract carcinogenesis, a group of collaborative projects will provide data to characterize, define, and standardize the conditions of exposure to respiratory carcinogens in the presence of vehicles and particles with different physico-chemical properties. — The kinetics of the cellular response to such exposures will also be determined. The relation between squamous differentiation and neoplastic response in the respiratory epithelium and their concurrent inhibition by vitamin A will be further characterized and defined. Several additional projects in the Carcinogenesis Area have direct relevance to the overall scope of the program.

I. INTRODUCTION

Cancer of the lung and bronchi has become a disease problem of increasingly alarming proportions. This year in the United States it will claim nearly 60,000

lives, mostly male cigarette smokers. As a disease entity, cancer of the lung and bronchi is in large measure responsible for the steady increase of total cancer mortality in the United States during the last decade.

In recognition of these facts, President Johnson in his health message of February 1967 called for the establishment of a Lung Cancer Task Force to determine how research could most effectively be directed toward solution of the many difficult problems posed by lung cancer. With the Director of the National Cancer Institute as chairman, a ten-member task force was constituted under the aegis of the National Cancer Institute. The physician and scientist members were drawn from government as well as private institutions. In addition, the following six organizations accepted invitations to appoint liaison members: American Cancer Society, Atomic Energy Commission, Department of Agriculture, American Medical Association, Federal Trade Commission, and The Council for Tobacco Research.

In the two 1967 meetings held in August and October, the Task Force considered and recommended a number of research alternatives. These fall generally into the categories of causation and prevention and of diagnosis and treatment. It was recommended that prime emphasis be given to the development of a less hazardous cigarette. A basic prerequisite for such an effort is the establishment of reliable bioassay and chemical assay systems through which cigarettes incorporating various tobaccos, filters, and other devices can be assessed and compared. It was also recommended that attention be given to environmental carcinogenesis and to research approaches to improve detection and diagnosis. Advisory work groups were to be appointed to study each of these areas in depth and to recommend the scientific efforts and resources necessary to implement a research plan. It was anticipated that specific programs could be initiated in fiscal year 1969.

Acting on the recommendations of the Task Force, the Director of the National Cancer Institute appointed a Working Group responsible for identifying and recommending programs of research which should lead to the development of less hazardous cigarettes. The members of the Group, which is designated the Tobacco Working Group (formerly the Less Hazardous Cigarette Working Group), represent numerous disciplines oriented principally to cancer. The membership includes the research directors for three of the major U.S. cigarette manufacturers, who bring to the task technical expertise and information of great importance.

It has been recognized that modifications of cigarettes to reduce one specific health hazard could possibly aggravate other health hazards. The National Institute of Environmental Health Sciences and the National Heart and Lung Institute are therefore represented on the Tobacco Working Group. Moreover, with the enlarged program scope of the National Heart and Lung Institute, considerations are being given to additional efforts in major disease categories, other than cancer, which have been associated with human exposure to cigarette smoke.

Responsibility for the operation of the Tobacco Working Group and for the implementation of research programs identified by the Group rests with the Scientific Director for Etiology, National Cancer Institute.

Early in the development of the Tobacco Working Group, each of the members was assigned to one of the following four Subgroups: (1) selection, preparation, and characterization of different types of tobacco and tobacco smoke condensates for subsequent bioassay and analysis; (2) bioassay of tobacco smoke and its fractions in a battery of animal systems; (3) chemical analysis of the tobacco smoke and its fractions; (4) evaluation of the data obtained in relation to the human situation.

These Subgroups have met separately and have reported their discussions and recommendations to the plenary sessions of the Tobacco Working Group.

The greatest need identified by the Group is the development of laboratory systems or models which could reliably indicate biological effects of tobacco smoke predictive of human response and which could be used to guide the tobacco technologists in the modification of cigarettes. The primary need is for biological models for respiratory carcinogenesis. Eventually, meaningful model systems should be developed for all disease categories which have been associated causally with human exposure to cigarette smoke.

Of about equal importance is the need for epidemiologic studies to correlate human disease experience with the response of animal model systems exposed to selected cigarette smoke components. Apart from helping to clarify the relevance of animal systems to the human problem, epidemiologic studies may help to identify components of cigarette smoke responsible for specific diseases and thus provide the basis for specific preventive measures.

Work on the causative factors and pathogenetic mechanisms of lung cancer had been previously included in the Carcinogenesis Program of the Etiology Area, NCI, through its intramural research activities, contract-supported collaborative programs, and an interagency agreement between NCI and the Atomic Energy Commission for the carcinogenesis program at the Oak Ridge National Laboratory.

A Lung Cancer Unit, directly responsible to the Associate Scientific Director for Carcinogenesis, has been established in the Etiology Area of NCI (effective January 1970).

The coordinated network of projects developed through all the programs mentioned above has received a high priority among the targeted research programs of NCI in the Carcinogenesis Area of Etiology and now constitutes its Special Lung Cancer Program.

An initial planning phase took place in the summer and fall of 1968. During that time two main efforts were made that contributed to the development of the Special Lung Cancer Program. On one hand the NCI outlined its "Program Plan on Chemical Carcinogenesis and Prevention of Cancers," which is addressed

to the entire issue of carcinogenesis and includes special consideration for the lung cancer problem; on the other hand, the Tobacco Working Group prepared a series of specific recommendations on its pertinent program needs.

An appropriation was made to the Lung Cancer Task Force for use in fiscal year 1969. From this appropriation, $1,153,000 was assigned to the Carcinogenesis Area, Etiology, to implement the initial phases of the program through contract-supported collaborative research.

Following the recommendations of the Tobacco Working Group, a balanced program has been developed to include selection, preparation, and analysis of different types of tobacco smoke condensates; bioassays on existing animal systems, particularly for topical carcinogenicity by mouse skin application and for cytotoxic and ciliary-inhibitory effects on *in vitro* and *in vivo* systems; and development of additional test systems for the bioassay of tobacco smoke condensate by ingestion and by direct administration into the respiratory tract. In view of striking observations recently obtained in the Carcinogenesis collaborative program, showing a key role of particulate materials in the respiratory tract for the manifestation of carcinogenic effects, an effort is being developed to characterize, define, and standardize the conditions of exposure to respiratory carcinogens in the presence of vehicles and particles with different physico-chemical characteristics. A study has been set up to obtain a definition of the kinetics of the cellular response to the respiratory administration of particulates and carcinogens. In addition, a study has been devoted to defining and characterizing the conditions responsible for the observed inhibition of squamous metaplasia and squamous-cell carcinoma development in the respiratory tract under the effect of vitamin A.

These new projects are coordinated with the on-going collaborative programs, in which studies related to the definition of bioassay models for respiratory carcinogenesis and co-carcinogenesis are being further extended.

II. THE PROGRAM PLAN ON CHEMICAL CARCINOGENESIS AND PREVENTION OF CANCERS

The coordinated research program of the National Cancer Institute on carcinogenesis by chemical and physical factors, and on prevention of cancers, is assigned to the Carcinogenesis Area of Etiology, which is responsible for its planning, implementation, and management. Historically, this activity developed from the Carcinogenesis Studies Branch established in March 1961 as part of the Field Studies Area for intramural and collaborative investigations on carcinogenic factors, particularly as hazards in the human environment. With the reorganization of NCI and the establishment of the Office of the Scientific Director for Etiology in January 1966, the Carcinogenesis Area was organized under the Office of the Associate Scientific Director for Carcinogenesis, with the

task of "planning and administering a program of basic and applied research in carcinogenesis leading to the identification or definition of environmental carcinogens, and to the elucidation of carcinogenesis mechanisms."

A contract-supported collaborative program has been developed since 1962 to respond to the need for extensive bioassay testing and screening of potential environmental chemical carcinogenic hazards. Additional contract support has been given to studies of chemical analytical methods for the identification of carcinogens and co-carcinogens, to studies for the development of bioassays using both *in vivo* and *in vitro* systems, and finally to a variety of fundamental research programs on the mechanisms of carcinogenesis.

The Program Plan on Chemical Carcinogenesis and Prevention of Cancers was developed in 1968 through an extensive series of planning sessions by the authors and Mr. Louis M. Carrese with the participation of Dr. Abraham Cantarow and Mr. Richard A. Terselic. The Report of the Discussion Groups on Chemical Carcinogenesis, coordinated by Dr. Cantarow for the National Advisory Cancer Council, served as an excellent basis for outlining the research needs in the field of carcinogenesis. The convergence technique was used to identify the major objectives and the linear array of subsequent phases leading to their implementation. An outline of the program plan is given in Fig. 1, and its main phases are discussed below. The program is articulated in a number of segments. The first segment deals with (a) the epidemiologic identification of population groups showing different risks to different types of cancer and (b) the characterization of individuals in these groups as to their environmental factors and their biological and functional parameters. The program then develops the following three distinct approaches:

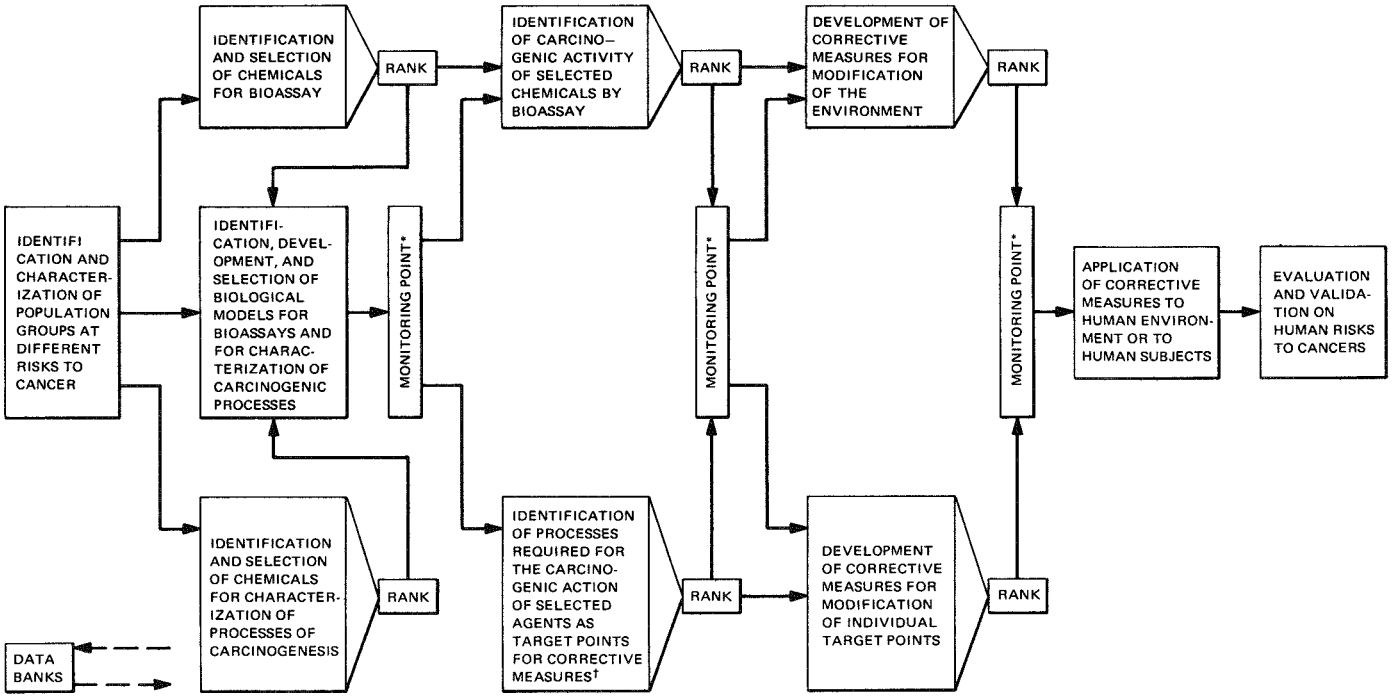
1. Identification of carcinogenic activity of selected chemicals by bioassay.

2. Identification, development, and selection of biological models for carcinogenesis bioassays as well as for the characterization of carcinogenesis processes.

3. Identification of processes required for the carcinogenic action of selected agents as target points for corrective measures.

The first two approaches require an initial phase devoted to the identification and selection of chemical agents to be entered in the bioassay systems or to be used for identification of steps in the carcinogenesis processes. The development and implementation of bioassay procedures requires a great deal of accurate standardization so that selected chemical agents will be tested through a battery of precisely defined and reproducible bioassay systems, each well-characterized for its sensitivity to specific carcinogenic effects.

After appropriate monitoring for relevance and for program needs, the results of the first phases of the program will lead to the development of corrective measures based, on the one hand, on modifications of the environment and, on the other hand, on modifications of individual target points in the



*Monitoring points According to program relevance and needs, determine further action and channel through next phases

†Through studies on the penetration of the agents into the organism, their transport, retention, and elimination, their metabolic conversion to the proximate carcinogen the cellular and tissue factors required for such processes, the penetration into target cells and interaction with cell constituents the neoplastic transformation, and the factors required for the growth of transformed cells into tumors

Fig. 1 – Chemical carcinogenesis and prevention of cancer (summary of program plans).

process of carcinogenesis identified by previous studies. These target points can be visualized as taking place at any one of the several steps required for the process of carcinogenesis to be completed. They include the following: (a) penetration of the chemical agent into the whole organisms; (b) transport, retention, and elimination of the chemicals in tissues (molecular logistics); (c) metabolic pathways to proximate carcinogen; (d) cell and tissue factors required for the previous steps; (e) penetration into target cells and interaction with cell constituents; (f) neoplastic transformation and conditioning factors; and (g) growth regulation of transformed cells.

The results thus obtained will be in turn appropriately monitored for relevance; the program then considers the development of corrective measures and ultimately suggests application of such corrective measures through the human environment or through the human subject. The last phase of the program deals with the evaluation and validation of the results of such corrective measures in the human situation and is expected to lead to the recognition of decreased cancer risks and in the populations originally studied in Phase I. Data banks will be organized for each of the major phases of the program and they will be a necessary source of information to be used for the monitoring of the whole program.

The program is aimed at a comprehensive attack on the problem of chemical carcinogenesis. It not only recognizes the need for very extensive bioassay efforts of an order of magnitude much greater than that presently available, but also recognizes that the accumulation of information on the carcinogenicity of chemicals in animal systems will not provide a direct extrapolation to the hazards in man. Moreover, it is predictable that a large number of chemical carcinogens will be difficult to remove and, therefore, will remain in the environment for a long time to come, even after being identified (e.g., combustion products, cigarette smoke, natural food contaminants such as mycotoxins, metals, and other air pollutants). It is therefore imperative that an effort be made not only to identify and possibly remove an increasing number of carcinogens from the environment, but also, and concurrently, to identify steps in their mode of penetration into the organisms and the target tissues and in their critical interaction with cell constituents, so that these steps could be exploited as target points for inhibitory or protective measures. The considerable development of refined technical methods presently available for the identification of these critical steps in carcinogenesis by studies in analytical chemistry, biochemistry, cell biology, molecular biology, and immunology can now be brought to bear on our ability to develop useful, protective measures against the ultimate effects of the carcinogenic processes. The identification of critical parameters required for the carcinogenic action of certain groups of chemicals, obtained in animal studies, also represents the only direct link that can be applied to the study of the susceptibility of man to comparable exposure conditions.

For example, the establishment of a strong correlation between the enzymatic activation of certain chemicals to the proximate carcinogen by means of given groups of enzymes, such as the microsomal aryl hydroxylases for polynuclear hydrocarbons, may lead to the identification of groups of individuals as being highly susceptible to certain specific carcinogenic exposures; pharmacologic inhibition of some of these enzyme systems may lead to a marked decrease of the cancer risk for such individuals. As other examples, analogous approaches can be made at the level of the control of protein synthesis in differentiation-dependent carcinogenic mechanisms (e.g., hormone-dependent tumors and possibly vitamin A-dependent tumors) and at the level of the immunological control of the growth of transformed cells. A broad front of attack can thus be developed against the sequence of events required for the carcinogenic process to lead to the establishment of progressively growing invasive tumors.

The effective development of such studies depends in part on the availability of appropriate animal models, capable of reproducing tumor types analogous to those observed in man. The development of appropriate biological models is particularly important for the establishment of selectively sensitive bioassay systems. An important phase of the program consists in the selection and standardization of bioassay systems that could be reproduced in several laboratories under strictly controlled standardized conditions. This is an essential requirement if we want to collect and analyze large series of data on carcinogenesis tests. For this purpose, a detailed plan is being worked out for a data retrieval and analysis system for carcinogenesis studies. The present overall Program Plan in Chemical Carcinogenesis and Prevention of Cancers constitutes the basis for the future development of detailed operational plans aimed at the implementation of each of its phases.

III. THE SPECIAL LUNG CANCER PROGRAM

The co-ordinated effort of the Carcinogenesis Area, Etiology, NCI, as directed towards the lung cancer problem, will be reviewed according to the phases in the conceptual plan in carcinogenesis studies outlined above (see Fig. 1).

A. Identification and Characterization of Population Groups at Different Risks to Cancer

1. An epidemiological planning study, presently underway, to establish the feasibility of an analysis of occupational and environmental factors associated with cancers detected by the Third National Cancer Survey and by other sources in the Detroit metropolitan area.[5] It is expected that lung cancer will constitute a large proportion of the cases studied by this project.

B. Identification and Selection of Chemicals for Bioassay and for Characterization of Processes of Carcinogenesis

1. Preparation and analysis of cigarette smoke condensate[20] from cigarettes of different characteristics, manufactured on the specifications recommended by the Tobacco Working Group. The condensates, prepared under standardized conditions, will be forwarded to other laboratories for bioassays.

2. Production and characterization of particulate materials for studies in respiratory carcinogenesis,[16] providing samples of a number of physicochemically characterized materials (e.g., carbon and various metal oxides) in the form of fine powders having a high degree of purity, a range of defined particle sizes, and defined surface activities. Known carcinogens will be attached to some of the particulates. The samples are then forwarded to other laboratories for biological studies on the role of particulates in respiratory carcinogenesis.

3. Analytical methods for selected tobacco smoke components.[2] A resource that will provide analytical support to the program for the development of a less hazardous cigarette, as recommended by the Tobacco Working Group, has been established. A coordinated set of analytical procedures has been devised and will be applied to the samples of cigarette smoke condensate used in this program.

4. Analytical methods for alkylating agents in tobacco smoke.[11] Such methods are now being developed with particular emphasis given to the detection of nitrosamines.

5. Synthesis, purification, and analytical methods for various classes of carcinogens.[6] Particular emphasis is placed on polynuclear hydrocarbons and *N*-nitroso compounds, which are prepared and characterized prior to biological study.

C. Identification, Development, and Selection of Biological Models for Bioassays and for Characterization of Carcinogenesis Processes

1. The development of the hamster intratracheal instillation model continues with studies on the definition of qualitative and quantitative responses of the hamster respiratory tract to polynuclear hydrocarbons and particulates, including dose-response studies and histopathological analysis.[6,22] Studies comparing different methods of direct administration of polynuclear hydrocarbons are also continuing.[7]

2. Definition of quantitative and qualitative responses of the respiratory tract in rats and hamsters to systemic administration of nitrosamines (e.g., diethylnitrosamine, *N*-nitrosoheptamethyleneimine, *N*-nitrosooctamethyleneimine) and to the intratracheal administration of nitrosamides.[6,22]

3. Role of particulates in respiratory carcinogenesis by intratracheal instillation. The cellular and tissue response of the different segments of the respiratory tract is studied following intratracheal instillation of suspensions of various particulate materials at different doses, alone or in combination with carcinogens.[6,22] Similarly, the reaction to materials of different and defined particle size and physico-chemical characteristics is analyzed.[17,21]

4. The role of solvents and vehicles in respiratory carcinogenesis is studied using the hamster intratracheal instillation model and determining both the morphological response and the distribution and retention rate of selected carcinogens in the presence of different vehicles and particles.[17,21] A technique for the administration of benzpyrene in an aqueous suspension without carrier dust[7] is used in studies on the role of particulates administered separately from the carcinogen.[6,7,17]

5. The induction of respiratory tract tumors by combined administration of different agents. The striking synergism demonstrated for the induction of lung tumors in hamsters by systemic pretreatment with diethylnitrosamine followed by intratracheal administration of ferric oxide[6,22] is being further studied and defined using different particulates, different doses, and different schedules of treatment.[6] The synergistic effect in hamsters primed with diethylnitrosamine is also studied after respiratory exposures obtained by inhalation of ozonized gasoline, ferric oxide or their combination, or of calcium chromate.[1]

   Further studies are devoted to the synergistic effect recently observed with irritant gases (SO_2, NO_2) and polynuclear hydrocarbons.[7] Other combined tests are listed as bioassays.

D. Identification of Carcinogenic Activity of Selected Chemicals by Bioassay

Bioassays are reported according to the mode of administration of the test material.

1. *Bioassays on the respiratory tract.*

   a. *Inhalation bioassays* include tests on the following materials: cigarette smoke in hamsters,[3] ozonized gasoline in mice and hamsters,[1] sulfur dioxide in rats and hamsters,[7] various halo ethers in rats and hamsters,[7] chromium oxide in mice,[1] calcium chromate in mice,[1] rats and hamsters,[3] ferric oxide in hamsters,[1] cobaltous oxide in hamsters,[3] nickelous oxide in hamsters,[3] and crysotile asbestos in hamsters.[3]

      Inhalation studies include tests for the combined effects of two or more inhaled materials – cigarette smoke combined with exposure to either asbestos or to a metal oxide;[3] sulfur dioxide combined with benzpyrene.[7]

b. *Intratracheal instillation bioassays* include: several polynuclear hydrocarbons (benzo[*a*]pyrene, dibenz[*a,i*]pyrene, benz[*a*]anthracene, 7,12-dimethylbenz[*a*]anthracene, dibenz[*a,h*]anthracene, dibenz[*a,h*]acridine, 7-*H*-dibenz[*c,g*]carbazole, benzo[*b*]fluoranthene) in saline suspension with a carrier dust;[6] other tests of polynuclear hydrocarbons (benzo[*a*]pyrene, 20-methylcholanthrene) use an aqueous suspension with some gelatine as vehicle.[7]

Several metal oxides and other particulates (ferric oxide, magnesium oxide, aluminum oxide, silicon dioxide as tridymite, cobaltous oxide, nickelous oxide, and carbon) are tested as saline suspensions in hamsters.[6] Arsenic trioxide is also tested in hamsters.[6] Four water-soluble nitrosamides (the *N*-methyl and *N*-ethyl derivatives of nitrosurea and nitrosourethane) are tested by intratracheal instillation in hamsters.[6] Tests of a polyester-fiberglass dust are being concluded.[7]

A pilot study is underway to determine conditions for testing tobacco smoke condensate by intratracheal instillation in hamsters.[6] Another pilot study is underway to determine test conditions for different chemical additives used in tobacco production.[6]

c. *Intrabronchial pellet implantation tests* have been developed using rats and hamster.[7] Current tests in rats include polyester-fiberglass, calcium chromate, and acid-washed cigarette smoke condensate in cholesterol.[7]

2. *Bioassays by routes other than respiratory.*

a. *Skin application tests* include a number of different samples of cigarette smoke condensate prepared according to standard specifications[20] and repeatedly applied to mouse skin in standardized test conditions.[14] This bioassay is the first of a battery of standardized tests for tobacco smoke to be implemented.

Several tobacco leaf extracts and fractions are tested for carcinogenic or promoting-effects on mouse skin.[8]

b. *Intramuscular injection tests* of several metal compounds (including organic derivatives) are continuing.[9] Several compounds that are strongly carcinogenic upon injection, such as titanium dioxide, will be selected for testing directly on the respiratory tract.

c. *Intraperitoneal implantation tests* are used to screen polyurethane plastics to determine if their breakdown products are carcinogenic.[12]

d. *Feeding tests* are used for the detection of systemic carcinogenic effects of cigarette smoke condensates[15] and of a variety of *N*-nitroso compounds.[6] Mice, rats, and hamsters are used for these studies.

Isonicotinic acid hydrazide (INH), the widely used antituberculosis drug, as well as isonicotinic acid and hydrazine sulfate, is being widely tested in mice, rats, and hamsters, in view of the reported induction of pulmonary adenomas in mice by hydrazine.[6]

e. *In vitro tests* are used for a study of the effects of tumor-promoting principles from tobacco on cell cultures.[8] Special bioassays for the determination of ciliary activity, mucous transport, and cytotoxicity of tobacco smoke are being used in the battery of tests applied to tobacco products.[13]

E. Identification of Processes Required in Carcinogenesis as Target Points for Corrective Action

A number of projects in the Carcinogenesis Area program are concerned with the identification of steps in the processes of carcinogenesis that could become targets for inhibition or prevention.

It is planned to make use of such scientific advances to generate specific projects aimed at the control of respiratory carcinogenesis.

The general areas of research that are under consideration in this respect include definition of the following:

1. The role of viral-chemical interactions in respiratory carcinogenesis.
2. The role of bacterial infections in respiratory carcinogenesis.
3. The pathogenetic role of immunologic factors.
4. The role of specific enzyme systems in the metabolic activation or inhibition of chemical carcinogens in the respiratory tract with particular attention to the microsomal aryl hydroxylases in the metabolism of polynuclear hydrocarbons.
5. The role of cell differentiation control mechanisms in carcinogenesis, with particular attention to the inhibitory effects of vitamin A. Studies of transplantability characteristics of tumors derived from different cell types in the respiratory tract will be continued. Morphological correlations in the histopathogenesis of respiratory tumors will be further explored by optical and electron microscopy and possibly correlated across species boundaries as well as *in vivo* and *in vitro* systems.

   An autoradiographic study of the kinetics of the response of the different cell types of the respiratory tract epithelium to administration of carcinogens and particulates is underway.[18]

   The role of vitamin A in the control of differentiation and carcinogenesis in the respiratory tract is studied in a collaborative program[19] and in the Carcinogenesis Area laboratories at NCI.

F. Information Bases and Data Analyses

1. *Information from the literature.*

   a. *Carcinogenesis Abstracts* has been reactivated and publications of this material will remain an on-going activity.[10]

   b. "Survey of Compounds Which Have Been Tested for Carcinogenic Activity,"[4] Supplement II (covering the years 1954–1960) of this series, will be published early in 1970. Material for the years 1968–1969 will be available late this year. Current plans call for publications of material covering the intervening years (1961–1967) in the Fall of 1971 and for continuation of the series in future years on a routine basis.

2. *Bioassay data system.* A comprehensive automated data system is being developed which will provide for routine systematic accumulation and storage of all pertinent data relating to carcinogenesis bioassay in a standard format. It will also provide for computer manipulation and recall of stored data by various parameters.

   The system is modular and will thus permit in-depth searches in specific data files. For example, "chemical file" is constructed so as to permit unique identification of agents, substructure searches, preferred and synonymous terminology, and cross reference to other files (Chemical Abstracts Service, National Library of Medicine, Cancer Chemotherapy National Screening Center, etc.).

   The system is designed to permit utilization of "canned programs" for statistical analyses of results. The system is "open-ended" and will allow for inclusion of other data (i.e., from the literature) as dictated by future program demands as well as inclusion of bioassay data from non-NCI-affiliated sources, provided such data are compatible with system format requirements.

3. *Conferences on Respiratory Carcinogenesis are being sponsored jointly with the U.S. Atomic Energy Commission.* The present Conference on Inhalation Carcinogenesis is the first one in this series, which is aimed at updating, reviewing, and bringing together scientific and technical information as a basis for further program planning.

   A Conference on Morphology of Experimental Respiratory Carcinogenesis is planned for May 1970.

REFERENCES

Carcinogenesis Contracts, Etiology, NCI

1. Atomic Energy Commission (FS-13): Co-Carcinogenesis Program.
2. Atomic Energy Commission (40-117-67): Collection, Separation, and Elucidation of the Components of Cigarette Smoke.

3. Battelle-Northwest Labs., Inc. (PH43-68-1372): Inhalation Studies.
4. John I. Thompson (PH43-69-2056): Detection, Exploration, and Quantitative and Synergistic Interactions among Chemical, Physical, and Biological Carcinogens under Controlled Environments in Avian and Murine Systems.
5. Michigan Cancer Research Foundation (PH43-69-2146): Planning Study on Cancer Incidence Correlated with Occupational History.
6. University of Nebraska (PH43-68-959): Carcinogenesis Research and Bioassays.
7. New York University (PH43-66-962): Inhalation Studies: Research in Carcinogenesis and Mechanisms of Anti-Carcinogenesis.
8. New York University (PH43-64-938): Studies of Carcinogenic Principals in Tobacco.
9. University of San Francisco (PH43-64-886): Studies of Carcinogenicity of Metallo-Organic Compounds.
10. Scientific Literature Corp. (PH43-70-2036): Carcinogenesis Abstracts, Vol. VIII.
11. Southwest Research Institute (PH43-65-1000): Evaluation of Free Radicals in Tobacco Smoke.
12. University of Tennessee (PH43-69-2077): Carcinogenesis Studies of Polyurethanes.

Lung Cancer Task Force Contracts, Etiology, NCI

13. A. D. Little, Inc. (PH43-69-2147): Bioassay of the Cytotoxicity of Cigarette Smoke and e Its Effects on Ciliary Function.
14. Hazleton Labs., Inc. (PH43-69-2149): Skin Carcinogenesis Bioassay of Cigarette Smoke Condensates in Mice.
15. Hazleton Labs., Inc. (PH43-69-2145): Carcinogenicity Bioassays by Intragastric Intubation of Cigarette Smoke Condensates in Experimental Animals.
16. IIT Research Institute (PH43-69-2075): Production and Characterization of Particulate Materials for Studies in Respiratory Carcinogenesis.
17. IIT Research Institute (PH43-69-2148): Role of Vehicles and Particulates in Respiratory Carcinogenesis Bioassay Using Intratracheal Instillation Techniques in Syrian Hamsters.
18. Marquette School of Medicine (PH43-69-2082): Autoradiographic Study of the Cellular Response of the Respiratory Tract in Chemical Carcinogenesis.
19. Massachusetts Institute of Technology (PH43-69-2083): Role of Vitamin A in the Control of Differentiation and Carcinogenesis in the Respiratory Tract.
20. Melpar, Inc. (PH43-69-2084): Preparation and Analysis of Cigarette Smoke Condensates Samples.
21. Ohio State Research Foundation (PH43-69-2144): Role of Vehicles and Particulates in Respiratory Carcinogenesis Bioassay using Intratracheal Instillation Techniques in Syrian Hamsters.

NCI, Intramural Program

22. Carcinogenesis Area, Etiology.

DISCUSSION

C. G. Baker: Thank you, Dr. Saffiotti. I will delay general discussion until all three of the panel members have presented their talks, but I would like to elaborate on the plans discussed by Dr. Saffiotti before going on to the next paper. As those of you who are interested in modern systems management will note, the conceptual base of the planning is the systems approach. This of course

leads quickly into very large areas of research efforts, since chemical carcinogenesis is of large and varied scope. There are hundreds of projects represented in the plan, some in being, some proposed. There are two important reasons for delineating plans in this manner: *One,* in order to establish priorities for allocation of resources, clearly stated, major objectives within some framework of the various aspects of carcinogenesis research must be delineated; and *two,* correlation of data from different experiments and projects requires a common groundwork, i.e., one system. This includes coordinated experimental designs. Without this systems approach, highly important correlations of several types cannot be made satisfactorily.

The effort outlined here was presented to the National Advisory Cancer Council as representing a program of a minimum of $20 million per year investment but one that could go as high as $100 million per year. We obviously are not operating programs anything like this, but we believe that the size of problems in carcinogenesis deserves this magnitude of effort.

Dr. Saffiotti also did not discuss the NCI grant-supported effort in carcinogenesis, which is in addition to what he was talking about. As many of you know, this area has not been as popular as some other areas of cancer research. The grant applications that arrive at the National Cancer Institute in the carcinogenesis area have been fairly steady over the years and have been funded at a level of only about $4 million or so, of which respiratory carcinogenesis is only a small part.

Much of this NCI planning was preceded by a study conducted by several panels of experts in conjunction with the National Advisory Cancer Council. The ideas represented in the plans are not just those of the staff of the Cancer Institute. Usually, when I say *we* (as I did in my earlier presentation) I mean all of us in this field totally, and not just the staff at the National Cancer Institute. How we bring together these complex areas and attempt to relate the animal data and the epidemological data remains one of our most challenging tasks.

PROGRAM PLANNING IN INHALATION CARCINOGENESIS: USAEC PLANS

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ABSTRACT

Although airborne radioactivity was implicated as a pulmonary carcinogen early in this century, we have known for several centuries that pitchblende miners develop fatal pulmonary diseases Perhaps less well known is the fact that ionizing radiation is one of the best tools available to the biologist for producing pulmonary tumors experimentally In this regard, studies using experimental animals may provide important links to studies in human beings — The AEC research program, conducted by the Division of Biology and Medicine, is mainly related to the following research efforts

1 Development of theoretical and empirical models to describe deposition retention, translocation and radiation dose-rate patterns of inhaled radionuclides within the lung and other tissues of interest

2 Investigation of early and late effects of several species of plutonium, other transuranic elements, selected fission products, and other selected materials (e g., rare-earth elements) following inhalation of various physical and chemical forms of these nuclides

3 Studies of inhaled constituents of uranium mine environments in experimental animals, this program is correlated with the uranium miners by comparative studies of sputum cytology and chemical analyses of lung samples from miners and experimental animals and by comparative measurement of constituents of mine air and animal exposure chambers

4 Studies of the cytokinetics of lung cells, macrophage function and transport of radioparticulates and the effects of pharmacologic agents on phagocytosis and clearance mechanisms

5 Development of feasible therapeutic means of reducing lung burdens of radioactive materials following deposition

This presentation will deal primarily with anticipated changes in the overall program occasioned by technological advances such as breeder reactors and the increased usage of thermoelectric generators for terrestrial, marine, and space applications

Almost a decade ago, in January 1960, the National Academy of Sciences subcommittee on Inhalation Hazards met in Richland, Washington, to prepare a draft of its final report on the "Effects of Inhaled Radioactive Particles" Among their conclusions and recommendations was a section entitled, "Research Needs," which is duplicated below [1]

1 Inhalation studies with specific radionuclides should be continued in several species of animals in order to better define deposition, retention, clearance, turnover, and biological effects

2 The influence of pre-existing pathological processes on deposition, clearance, and retention of particles in both upper and lower respiratory tract should be determined

3 Information is needed on the relative biological effectiveness of various radiations in causing late injury such as cancer

4 Possible synergism between radiation and chemicals should be studied

5 Better instrumentation is needed for measurements of solubility, and of particle size and distribution of radioactivity on particles in the 0 05- to 0 5-micron size range, in order to correlate physical properties with observed biological effects The instruments should be usable in the field as well as in the laboratory

6 More information should be obtained on the physical and chemical properties of small particles

7 Better methods are required to estimate lung burdens in humans

8 Programs should be considered for measuring concentrations of radionuclides in human tissue where there is reason to believe there was significant exposure to radioactive particles

9 The least effectively controlled inhalation hazards from radioactive materials appear to be those associated with radon and daughter products in mining operations Efforts to control this hazard should be intensified

10 Since knowledge of the effects of inhaled materials is rapidly expanding, reconsideration of these recommendations should be made from time to time as new information is acquired

With only minor alterations these research needs still represent a reasonable description of the Atomic Energy Commission's internal emitter program as related to the inhalation route of exposure Those of you who toil in such fields are acutely, and perhaps painfully, aware that progress is made slowly and often with much difficulty More specifically, the AEC's program in inhalation carcinogenesis encompasses the following general research areas

1 Development of theoretical and empirical models to describe deposition, retention, translocation, and radiation dose-rate patterns of inhaled radionuclides within the lung and other tissues of interest

2 Investigation of early and late effects of several species of plutonium, other transuranic elements, selected fission products, and other selected materials (e.g., rare-earth elements) following inhalation of various physical and chemical forms

3. Study of inhaled constituents of uranium mine environments in experimental animals; this program is correlated with the uranium miners by comparative studies of sputum cytology and chemical analyses of lung samples from miners and experimental animals and by comparative measurement of constituents of mine air and animal exposure chambers.
4. Investigation of the cytokinetics of lung cells, macrophage function and transport of radioparticulates and the effects of pharmacologic agents on phagocytosis and clearance mechanisms.
5. Development of feasible therapeutic means of reducing lung burdens of radioactive materials following deposition.

Many reasons exist as to why it is important to conduct this program. Aside from the obvious relation to personnel protection within the nuclear energy industry, much of the information obtained from the program is applicable to the more general problem of environmental pollution from nonradioactive materials. As regards radiation protection, there is a very cogent reason for studying the effect of inhaled radioactive materials. Radiation protection standards and criteria for bone-seeking radionuclides, as promulgated by organizations such as the National Council for Radiation Protection and Measurements (NCRP) and the International Commission on Radiation Protection (ICRP), actually are based on a dual system: The observations based upon the human radium recipients and "allowable" exposure estimates which can be related to multiples of the radiation dose-rate arising from natural background. In fact, it is encouraging that recently recommended allowable dose-rates for specific regions or cell types (e.g., marrow, endosteal surface) in bone[2] appear to support the long-used values based either directly or indirectly on the 0.1 μCi ^{226}Ra standard.

The AEC's central program for the bone-seeking radionuclides is built around a large-scale retrospective epidemiological study of radium toxicity involving several thousand people who were exposed through employment (mostly radium dial painters) or medical treatment. These studies serve as a base line of intercomparison for comparative experiments in which the toxicity and relative hazard of other bone-seeking radionuclides are assessed through the following relation:

$$\frac{\text{Effect of } ^{239}\text{Pu on man}}{\text{Effect of } ^{226}\text{Ra on man}} \cong \frac{\text{Effect of } ^{239}\text{Pu on experimental animal}}{\text{Effect of } ^{226}\text{Ra on experimental animal}}$$

Thus, the toxicity of ^{226}Ra and ^{239}Pu can be determined in animals and, because the toxicity of ^{226}Ra in man is known, the toxicity of ^{239}Pu in man can be estimated.

No such standard based upon human data exists for the lung or for soft tissues in general. Also, it is unlikely for many reasons that information obtained

from uranium miners will provide a counterpart to the information obtained from the human radium recipients.

Over the years our collective experience has indicated that inhalation as a route of exposure to radioactive (and stable) materials in industry must be assigned a high probability. Large projected increases in nuclear power production with attendant increases in nuclear fuel manufacture and processing indicate the need and justification for the program outlined above. Another consideration, despite the very low probabilities of release, is that of high specific-activity radioparticulates from nuclear propulsion reactors and thermoelectric generators* designed for numerous uses. Paralleling these developments will be greatly expanded production of uranium ore, the majority of which will come from mines rather than from open pit sources.

As pointed out by Furth and Tullis,[3] thorium dioxide was perhaps the best available carcinogen prior to the discovery of the carcinogenic hydrocarbons. Among the tumors reported from the use of thorium dioxide, Abrahamson *et al.*[4] reported a carcinoma of the lung some 18 years after bronchography with thorium dioxide; numerous carcinomas and sarcomas were induced in various species. The basic findings on the relative responsiveness of various tissues to thorium dioxide were later duplicated with hydrocarbons and with artificially produced radionuclides. Cember's[5] review of radiogenic lung cancer and the National Academy of Sciences–National Research Council report[6] on the effects of inhaled radioactive particles provide good summaries on the types of experimental pulmonary lung cancer produced by radioactive substances. Since then, descriptions of broncheolar carcinoma produced by plutonium in dogs have been reported.[7-8] Sanders *et al.*[9] recently reviewed data on lung cancer incidence in experimental animals arising from the administration of alpha- or beta-gamma-emitting radionuclides. The use of radioactivity provides the biologist with an efficient means of producing pulmonary lesions, including neoplasia, in experimental animals.

The remainder of this presentation identifies most of the current projects related to the inhalation phase of the internal emitter program conducted by various contractors for the Division of Biology and Medicine of the AEC. Descriptions of many of the projects presented here are available from an AEC document entitled "Research and Development in Progress – Biology and Medicine."[10] Listed below, for convenience, are the abbreviations of the laboratories conducting these projects:

ANL — Argonne National Laboratory, Argonne, Illinois

CSU — Colorado State University, Fort Collins, Colorado

HASL — AEC Health and Safety Laboratory, New York, New York

HEHF — Hanford Environmental Health Foundation, Richland, Washington

*SNAP, or Systems for Nuclear Auxilliary Power.

LASL — Los Alamos Scientific Laboratory, Los Alamos, New Mexico

LF — Lovelace Foundation, Albuquerque, New Mexico

LRL L — Lawrence Radiation Laboratory, Livermore, California

MIT — Massachusetts Institute of Technology Cambridge, Massachusetts

ORNL — Oak Ridge National Laboratory, Oak Ridge, Tennessee

PNL — Pacific Northwest Laboratory, Battelle Memorial Institute, Richland, Washington

SMH — St Mary's Hospital, Grand Junction, Colorado

UC — University of Connecticut, Hartford, Connecticut

UH — University of Hawaii, Honolulu, Hawaii

UP — University of Pittsburgh, Pittsburgh, Pennsylvania

URAEP — University of Rochester Atomic Energy Project, Rochester, New York

WSU — Wayne State University, Detroit, Michigan

1 Development of theoretical and empirical models to describe deposition, retention, translocation, and radiation dose-rate patterns of inhaled radionuclides within the lung and other tissues of interest Several laboratories are developing sophisticated models, often with the assistance of analog and digital computers, to describe lung dynamics, translocation, and retention of radionuclides for purposes of radiation protection and describing particle dynamics Plutonium-239 excretion data, obtained from human patients given $^{239}Pu(NO_3)_4$ complexed with citrate about 20 years ago, still provide the main basis for interpreting human exposures to plutonium Since the publication of the International Commission on Radiological Protection (ICRP) report by the Task Group on Lung Dynamics,[11] the human plutonium data have been incorporated into the new ICRP lung model to describe more accurately cases of plutonium inhalation (ORNL, LASL, URAEP, PNL, LRL-L). Another model was devised to predict the dose to the respiratory tract from continuous inhalation of radioactive aerosols (URAEP) At PNL, animal data collected during the past 11 years were used in models that simulate inhalation of plutonium oxide by dogs These models provide a valuable adjunct to the experimental data. For example, the model predicts maximum bone and liver levels of 2 to 3% of the original amount of $^{239}PuO_2$ inhaled by beagles From this model one can estimate the decrease in the tracheobronchial lymph-node burden as a function of time beyond the period for which experimental data were obtained

At URAEP a lung model capable of describing distributional phenomena is being developed. It incorporates a mathematical description of the mechanical behavior of a continuous tissue matrix with spatially varying permeability consistent with Weibel's data for the human bronchial tree

A cubical lattice model of the "deep respiratory zone" was developed at LRL-L and used to assess the carcinogenic risk from insoluble alpha-emitting

aerosols deposited in deep respiratory tissue. Another model, based upon tumor probability estimates obtained from experiments in which rat skin is irradiated, was developed at LASL. It incorporates parameters such as particle size and number, velocity of the particles, and residence time within the lung. One version of this model assumes that each particle moves at a constant velocity from the bottom of an alveolus to the ciliated epithelium during 720 days. However, rat skin tumor formation is strongly affected by spatial distribution of the radiation dose in the skin and on the linear energy transfer of the ionizing radiation. Also, the rat skin data is probably a poor source of input to the model because of the relative ease with which skin tumors are produced and the existence of a critical depth for tumorigenesis.

A program that makes use of hybrid computer techniques was developed at PNL to describe the distribution of alpha particle energy around plutonium particles with alveoli. About 60% of the alpha energy is absorbed by the alveolus harboring the particle. The alveolar epithelium is partly shielded from the alpha irradiation after macrophage incorporation of the plutonium particle.

*2. Investigations of early and late effects of several species of plutonium, other transuranic elements, selected fission products, and other selected materials (*e.g., *rare-earth elements) following inhalation of various physical and chemical forms.* Biological studies of alpha emitters, particularly isotopes of the actinide series, has been a major importance at URAEP. Inhalation studies with $^{239}PuO_2$, started in 1957, have been completed. A smaller study with $^{238}PuO_2$ has also been completed. The latter studies were designed to examine radiation effects, vis-a-vis, and chemical effects, and to obtain additional data on pathological effects, particularly the progressive lymphopenia seen in most dogs. Pulmonary retention, clearance, and biological effects, including cytological and histopathological responses, have also been investigated. The average half-time for pulmonary clearance was found to be 400 days in these experiments.

Inhaled plutonium is also the subject of investigation at PNL. Radionuclides studied include ^{237}Pu, ^{238}Pu, ^{239}Pu, ^{240}Pu, ^{241}Pu, and ^{242}Pu; compounds investigated include PuO_2, $Pu[NO_3]_4$, and PuF_4. These investigations are designed to investigate both acute and chronic biological effects, including the development of fibrosis and tumor formation.

The results of one study in approximately 40 dogs, started 10 years ago, clearly shows the relation between the amount of plutonium deposited and survival time. Life-shortening is expected in animals that receive alveolar depositions of more than ~5 nCi of plutonium per gram of lung. The lung cancer incidence has been high in this experiment, as indicated by Dr. Sanders (see these *Proceedings*, pp. 284–286). Of these survivors, only a few showed neither radiographic evidence of a lung tumor nor severe lymphopenia. A lung tumor formation has always followed a marked sustained lymphopenia. More recently, PNL has conducted inhalation experiments with high specific activity ^{238}Pu both as aerosols and as microspheres. Extensive histological descriptions of induced lesions are an important end point in these investigations.

At LF in Albuquerque, studies have been in progress for a number of years to determine the biological effects of fission product radionuclides deposited in the lung. These investigations include the study of the distribution and excretion, as well as the toxicity and pathology, of these fission products. Both soluble and insoluble forms of ^{90}Sr, ^{131}I, ^{144}Ce, ^{137}Cs, ^{106}Ru are studied at several exposure levels in beagles. These experiments are part of a long-term study of the biological consequences of inhaling fission products radionuclides such as might be released from a nuclear accident. Animals are exposed once via inhalation to an aerosol contaminated with sufficient radionuclide activity to produce biological damage or shortening of life-span. Biochemical, hematological, clinical, and microbiological changes which might be attributed to the radiation insult are followed throughout the remainder of the animal's life-span; pathological examinations are performed at death. Supporting the experiment at LF are investigations such as the secondary antibody response in beagles following inhalation; effects of inhalation of fission products on the composition and invasiveness of the microbial flora of the nose, throat, and lower intestinal tract of beagles; and the effect of inhaled fission products on the antibacterial activity of beagle serum. To date most of the experience of the LF group has been with soluble forms of the radionuclides under study, but more recently emphasis has been directed toward insoluble forms. In addition, the LF group is now initiating studies with alpha-emitting radionuclides such as $^{241}AmO_2$.

New applications of rare earth compounds have stimulated the collection of further toxicologic data on these materials at URAEP. The objective of this research is to determine the health hazards associated with the inhalation of a number of airborne rare earth elements. In the first series of studies, beagles are being exposed to an aerosol of europium oxide (EU_2O_3) having a particle size of about 0.5 μ mass median diameter (MMD). Both single and multiple exposure will be conducted at air concentrations ranging from five to several hundred milligrams of Eu per cubic meter of air. Exposed animals and control groups will be examined routinely for changes in general health, body weight, hematology, and histology. Concentrations of Eu in critical organs and excreta are determined by activation analysis. Investigations in the future will include the study of the oxides and possibly the soluble salts of other rare earth elements.

Another study, at WSU, deals with the short-term metabolism and excretion, by the Sprague-Dawley rat, of yttrium, scandium, and a few rare earths representative of light, intermediate, and heavy groups (e.g., cerium, europium, and ytterbium). Major emphasis will be directed towards obtaining data on inhaled aerosols of the stable element acting as a vector for the corresponding radionuclide. The residence time in the lung for the various rare earths used will be determined. Although the animal response is not a prime consideration in these studies, selected tissues will be sampled for possible detection of pathological conditions especially in the lung.

The toxicology of nickel carbonyl, one of the most toxic chemicals encountered in industrial operations, is being investigated at UC. The hazard of

nickel carbonyl inhalation is prevalent in the chemical, oil, rubber, electronics, and metallurgical industries.

Chronic exposures to nickel carbonyl have been implicated as a causative factor in pulmonary carcinogenesis. Comparisons are made of acute and chronic toxicity and carcinogenicity of nickel carbonyl in rats by inhalation and by parenteral injections at several dose levels. By use of radioactive ^{63}Ni carbonyl, precise information is obtained on the organ distribution and rates of excretion of nickel carbonyl. Sprague-Dawley rats are treated with $Ni(CO)_4$ and $^{63}Ni(CO)_4$. The acute clinical and pathological reactions to parenteral $Ni(CO)_4$ develop primarily in the lungs, resembling the reactions which were previously observed following inhalation of $Ni(CO)_4$. Experiments with $^{63}Ni(CO)_4$ have revealed that parenterally administered $Ni(CO)_4$ is partially excreted in the expired air. Nickel carbonyl has been found to interfere in the induction of enzymes in the lung. Current studies suggest that this inhibition of enzyme induction is mediated by binding of Ni to messenger RNA.

Last, but certainly not least, is the Carcinogenesis Program at ORNL. This research, which attempts to study the effects of radiation, virus, and chemicals as carcinogens as well as cocarcinogens, is jointly sponsored by the National Cancer Institute and the AEC.

3. Studies of inhaled constituents of uranium mine environments and experimental animals. This program is correlated with the uranium miners by comparative studies of sputum cytology in chemical analyses of lung samples from miners and experimental animals and by comparative measurements of constituents of mine air and animal exposure chambers.

The radiobiology of radon and its progeny has been studied in rodents for some years at URAEP. More recently, studies of radon and its progeny have been undertaken in dogs. Four dose levels are being studied in 30 dogs, covering the range of 250–3800 working level months (WLM). Average concentrations of radon in its decay products are: radon, 0.6 μCi/liter; radium A, 0.45 μCi/liter; radium B, 0.3 μCi/liter; and radium C-C′, 0.18 μCi/liter. Exposures are 20 hr per day for the required number of days, at the rate of 5 days per week, until the desired exposures are reached. The radon decay products are carried on normal room dust in this experiment. This work is being conducted in conjunction with the National Institute of Environmental and Health Sciences of the U.S. Public Health Service. Additional exposures are planned as part of this experiment.

Related studies, some also jointly funded with the USPHS, are under way at PNL. This program is also designed to investigate possible causative factors in the observed increased incidence of lung cancer among uranium miners. One portion of the experiment involves a study of the long-term biological separation of long-lived alpha emitters in the uranium decay chain. Samples of lung tissue from deceased miners are analyzed for numerous nuclides by neutron activation analysis. The elements scandium and antimony, typically high in concentration among individuals with mining experience, were found to be low in nonminer lung samples. Similar studies are being conducted at ANL.

In addition, hamsters are exposed to various combinations of uranium ore dusts, and radon and its progeny. Histopathological examination of respiratory tissue from hamsters in the low exposure group (32 WL), 9 months after initiation of exposures, are now being conducted. Supporting experiments with beagles are also designed at PNL and will include exposure to radon and its progeny plus combinations of uranium ore, diesel exhaust fumes, and tobacco smoke. Dr. Stuart from PNL presented this material to us earlier in the Conference (see Bruce O. Stuart *et al.*, pp. 419–424).

A study at CSU is directed towards filling the gap between the known biological hazards from inhalation of radon progeny and other mine air constituents and appropriate methods for evaluation and control of exposure to mine atmospheres. Human subjects are exposed to mine aerosols both in a controlled test tunnel and in general working areas in an operating uranium mine. Chemical characteristics of the exposure aerosols are measured from samples collected concurrently with exposures. Deposition patterns of radon progeny in the human respiratory tracts are quantitated by equipment in a mobile whole-body counting laboratory. Samples of urine, blood, and hair are routinely collected from miners and analyzed for long-lived radon progeny. Depositions and resultant radiation doses are studied with respect to the various mine atmospheres. Data from the mine under study indicate that less than one-third of the airborne radon progeny is present in uncombined atoms or ions; the remaining fraction is generally attached to particles in the submicron range.

The relationship between physical properties of inhaled aerosols and respiratory deposition under controlled exposures is being studied at HASL. Human respiratory deposition is being related to aerosol particle size, density and charge and to breathing patterns in experiments designed to check the validity of current lung models and to extend the present range of numerical data. In another phase of this work simultaneous measurements of human respiratory deposition and the concentration, particle size, and charge of aerosols are being made to define the significant factors relating occupation exposure to hazard. In the relationship of aerosol properties, respiratory deposition and excretion are measured in actual work atmospheres. Field studies have been initiated in a uranium mine and in a uranium fabrication plant.

The dosimetry of selected tissues in radium workers as applied to lung carcinoma in uranium workers is under investigation at MIT. The unusually high incidence of carcinomas in the sinus cavities of radium recipients suggest the importance of obtaining the quantitative estimate of the dose rate to the overlaying epithelial tissues of the sinus cavities from the presence of radium and its progeny in and around the cavity. It is hoped that results of this dosimetry study will be applicable to the problem of lung carcinomas in uranium miners.

An investigation being conducted at SMH is designed to study sputum cytology from uranium miners with and without past smoking history. Out-growths of this project will be the development of uranium miner tumor registry and an atlas describing sputum cytology. In an attempt to integrate

these observations with experimental data, lung washings from dogs containing Pu or radon progeny are obtained prior to dog sacrifice at PNL and compared with samples obtained from uranium miners and from confirmed cases of pulmonary neoplasia.

The fate of inhaled ^{210}Pb in human subjects is being investigated at URAEP. This study is related not only to the uranium mining problem as regards long-lived radon progeny such as ^{210}Pb, but also to the general problem of lead contamination in the environment.

4. Studies of the cytokinetics of lung cells, macrophage function and transport of radioparticulates and the effects of pharmacologic agents on phagocytosis and clearance mechanisms. The objective of experiments at URAEP is to correlate structural and functional changes following aerosol inhalation by means of light and electron microscopy. Autoradiographic and histochemical techniques are also used. Particular emphasis is placed on the role of phagocytosis in the clearance of insoluble particulates from the lung. The origin of free alveolar macrophages and mechanisms of transfer of particulates to interstitial and lymphoid tissue are also studied.

At PNL the maintenance of phagocytic function following $^{239}PuO_2$ administration is studied. Latex beads are used as a source of ingested material for the macrophages. Electron microscopy is used in related macrophage function studies.

Other work related to this category is conducted at UH.

5. Development of feasible therapeutic means of reducing lung burdens of radioactive materials following deposition. Critical safety analyses conducted for a number of nuclear operations confirm that the primary hazard for potential accidents for a number of operations is related to inhalation of radionuclides that are accidentally released. Fortunately, the number of individuals who have inhaled and deposited significant amounts of radioactive materials in their lungs is small. Nonetheless, the rapid growth of the nuclear industry increases the potential for accidental exposures at levels that may warrant efforts to remove the radioactive material deposited in the lungs, thereby reducing the radiation exposure that would result from the deposited radioactive material and potentially preventing radiation-induced disease.

A project at LF is specifically directed to developing improved procedures for reducing the lung and, therefore, body burden of inhaled radionuclides. Major attention has been directed to the development and use of a broncho-pulmonary lavage procedure for removing inhaled radionuclides from the lungs of beagle dogs. Future work will be directed toward improving this technique through changes in the lavage fluid including electrolyte composition, addition of pharmacologic agents such as bronchodilating and mucolytic agents, and addition of chelating agents for some radionuclides. Experiments to date with several radionuclides have shown encouraging results.

Pulmonary lavage has also been used on excised rat lungs at PNL. Nearly all the ^{239}Pu particles removed the first day after inhalation exposure had been

phagocytized by pulmonary macrophages. Removal efficiency appears to be inversely related to lung pathology.

At PNL, rats were exposed to $^{239}PuO_2$ aerosols and subsequently treated with negatively charged electro-aerosols to establish their effect on pulmonary clearance of inhaled plutonium. In general, the treated groups had lower average ^{239}Pu lung burdens at sacrifice than control groups treated with uncharged aerosols. Others have observed that negative air ions significantly accelerate ciliary activity for prolonged times in both experimental animals and man.

Injection of the chelating agent DTPA caused no significant increase in the clearance rate of plutonium from dogs exposed to $^{239}PuF_4$ aerosols at PNL. Pharmacological agents which might accelerate normal clearance patterns are also studied at PNL. Diuretics, tranquilizers, steroids, and antihistamines are among the agents tested. Chloropromazine, progesterone, and pheneragan have been among the most promising of these.

In addition to the above program, epidemiological studies related to exposures in the beryllium (MIT) and nuclear (UP) industries are in progress, and a national plutonium registry has been established at HEHF.

Major emphasis in AEC program planning as related to inhalation carcinogenesis. Two major efforts will be emphasized in the AEC program planning. The first is designed to extend the dose-response studies for plutonium and other transuranic radionuclides inhaled by dogs to lower initial depositions. It is important to obtain more quantitative information on the dose-response curve for life-shortening and tumorigenesis, especially at the low dose end. These long-term dog studies will involve several transuranics of varying physical properties. Table 1 shows selected physical characteristics for several transuranics. All are energetic alpha emitters which also emit gamma and X-radiations. The X- and gamma radiations provide a means of *in vivo* detection and assay in some cases. A wide range in specific activity is apparent.

Table 2 shows maximum permissible air concentrations for continuous occupational exposure to these radionuclides; the maximum permissible bone burdens are also given both as activity and mass. Although the International Commission on Radiological Protection does not list "lung burdens" for these materials, a value of .016 μCi for ^{238}Pu and ^{239}Pu has gained widespread acceptance. This brings us to the second major effort, the problem of nonhomogeneous distribution of dose as regards radiation protection guides and tumorigenesis.

From Table 3, one can estimate that 180 particles of $^{238}PuO_2$ would represent a 0.016 μCi lung burden that would deliver 15 rem per year initially to the lung. This dose, however, would be delivered to a relatively small number of cells as compared with the number of cells that would be exposed to the same activity contained in particles smaller than 1 μ diameter.

Table 4 shows the relation between particle diameter, particle number, disintegration rate, and number of cells irradiated for a lung burden of .016 μCi comprised of different particle sizes. As particle size decreases, for the same total

TABLE 1

Selected Physical Properties of Some Transuranic Radionuclides

Property	Radionuclide				
	^{238}Pu	^{239}Pu	^{241}Am	^{244}Cm	^{252}Cf
Physical half-life (yr)	89	24,360	458	18.1	2.65
Alpha energy (Mev)	5.5	5.1	5.5	5.8	6.1
Grams per curie	.058	16.1	0.29	.012	.0019
Curies per gram	17.18	.062	3.2	83.3	536
Power density (watts/g)	0.55	~.0002	0.11	2.8	38.5
L X-rays (% abundance)	U (13)	U (4)	Np (27)	Pu (8)	Cm (6)
Gamma rays (kev)	44, 99, 150	39, 52, 129, 207, 375, 414, 650, 770	26, 33, 43, 60, 101, 120, 160, 208, 335, 370, 663, 722	43, 100, 150, 262, 590, 820	43, 100, 160

TABLE 2

Maximum Permissible Concentrations for Air (MPC_a) and Maximum Permissible Body Burdens (q) for Several Transuranic Radionuclides

	MPC_a		q(Bone)	
	Bone	Lung	(μCi)	(μg)
^{238}Pu	7×10^{-12}	10×10^{-12}	04	$2\,4 \times 10^{-3}$
^{239}Pu	6×10^{-12}	10×10^{-12}	04	0 65
^{241}Am	2×10^{-12}	40×10^{-12}	0 1	15×10^{-3}
^{244}Cm	3×10^{-12}	30×10^{-12}	0 1	$1\,2 \times 10^{-3}$
^{252}Cf	2×10^{-12}	10×10^{-12}	0 01	19×10^{-6}

TABLE 3

Selected Physical Parameters for One Micron-Diameter Particles of Transuranic Radionuclides in the Dioxide Form

Nuclide	Physical half-life (yr)	Theoretical density (for oxide)	Volume (cm^3)	Specific activity (Ci/g)	Activity (pCi)
^{238}Pu	89	11 5	52×10^{-12}	14 6	88
^{239}Pu	24×10^3	11 5	52×10^{-12}	55×10^{-3}	33
^{241}Am	458×10^3	11 7	52×10^{-12}	2 6	16
^{242}Cm	46	11 7	52×10^{-12}	$2\,9 \times 10^3$	17.7×10^3
^{244}Cm	18	11 7	52×10^{-12}	70	42×10^3

TABLE 4

Relation Between Particle Diameter, Particle Number, Disintegration Rate and Number of Cells Irradiated for a Lung Burden of 0 016 μCi $^{238}PuO_2$

Diameter (μm)	Number of particles	Disintegration rate ($d \cdot week^{-1} \cdot particle^{-1}$)	Number of cells irradiated*
0 01	$0\,2 \times 10^9$	1 8	52×10^9
0 1	$0\,2 \times 10^6$	$1\,8 \times 10^3$	54×10^6
1 0	$0\,2 \times 10^3$	$1\,8 \times 10^6$	56×10^3

*For each particle size the number of cells exposed within a 40 μm alpha particle range is estimated The total number of cells irradiated becomes the product of the number of cells irradiated per particle and the number of particles A cell volume of 10^3 μm^3 is assumed

activity, more cells are exposed but at lower dose rates Experimental emphasis will be on particle size and number Both dogs and hamsters will be used These studies will also require monodisperse aerosols

In one study, particles will be introduced into the lung vasculature following intravenous injection and passage through the right heart This method of simulating deep lung deposition has been used successfully at LASL for single 150 200 μ particles

These studies will attempt to answer the general problem of tissue response (primarily lung) to insoluble inhaled radionuclides

REFERENCES

1 The Biological Effects of Atomic Radiation – Summary Reports, 1960, p 38 National Academy of Sciences, National Research Council, Washington, D C , 1960
2 *A Review of the Radiosensitivity of the Tissues in Bone* International Commission on Radiation Protection on Radiological Protection, Publication 11 Pergamon Press, London, 1968
3 J Furth and J L Tullis, Carcinogenesis by radioactive substances *Cancer Res*, 16 (1956) 5–21
4 L Abrahamson, M H O'Connor, and M L Abrahamson, Bilateral alveolar lung carcinoma associated with the injection of thorotrast *Irish J Med Sci*, 6 (1950) 229 235
5 Herman Cember, Radiogenic Lung Cancer *Progr Exptl Tumor Res*, 4 (1964) 251–303
6 Report of the Subcommittee on Inhalation Hazards, The Effects of Inhaled Radioactive Particles, Publication No 848 National Academy of Sciences, National Research Council Washington, D C (1961)
7 W J Clarke and W J Bair, Plutonium inhalation studies VI pathologic effects of inhaled plutonium particles in dogs *Health Phys* 10 (1964) 391–398
8 W J Clarke, J F Park, J L Palotay, and W J Bair, Plutonium inhalation studies VII Bronchiolo alveolar carcinomas of the canine lung following plutonium particle inhalation *Health Phys*, 12 (1966) 609–613
9 Charles L Sanders, Roy C Thompson and W J Bair, Carcinogenesis in the Lung from Inhalation of Radioactive Particles, USNRDL Report, NRDL-TRC-69-8, 18 March 1969
10 Research and Development in Progress – Biology and Medicine, USAEC-DTI, TID-4060, 1968
11 Task Group on Lung Dynamics, Deposition and retention models for internal dosimetry of the human respiratory tract *Health Phys*, 12 (1966) 173–207

DISCUSSION

C. G. Baker Thank you Dr Richmond Some areas of AEC program interest shown on Dr Richmond's slides might appear to overlap some of the areas covered by Dr Saffiotti, but even if there is some duplication, it is healthy From the standpoint of your interests in sources of funding, I am sure you appreciate the healthiness of it! We will have more trouble in maintaining this valued option if the "negative increases" in funding that Dr Richmond mentioned should continue much further

I would like to turn next to Dr. William Payne, who is Deputy Director of the National Institute of Environmental Health Sciences. As you may perceive from the title of that Institute, possible overlap exists with the research interests of other agencies, because environmental health sciences touch many aspects of other parts of NIH, and some other parts of DHEW. Some people worry about the lack of a sharp distinction between these different missions, but I believe the varied approaches are sound. The scope of these research areas is so vast that there is room for everybody in the problems. As in the case of NCI, the NIEHS staff are experts in carcinogenesis, but so as long as each of us keeps track of what the other is doing, no serious operational problems are likely.

PROGRAM OF THE NATIONAL INSTITUTE OF ENVIRONMENTAL HEALTH SCIENCES

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ABSTRACT

Established only recently, the National Institute of Environmental Health Sciences is located not in Bethesda but in Research Triangle Park, North Carolina Proximity to the three universities forming the Triangle makes possible a number of cooperative research and training ventures — Although its program is still in the initial stages, the mission of NIEHS is to determine and evaluate the health hazards of chemical, physical, and biological agents in the environment Through identification of the mechanisms in adverse response, criteria for protective or preventive measures can be established on a scientific basis Experimental data, clinical studies, and epidemiological observations are being developed into a structured, coordinated program to determine the pathogenesis of adverse effects and to evaluate exposure in relation to these effects A program of epidemiology and biometry is being developed to help bridge the gap between the laboratory and man, meanwhile, in the laboratory, the effects of environmental agents in small animals and various *in vivo* and *in vitro* systems are being studied under the full range of medical, biological, and physical sciences — An important but as yet undeveloped aspect of the program is the study of inhalation or whole-body exposure to toxic environmental agents, investigations by such means will be possible when expanded facilities are complete

The Oak Ridge National Laboratory, as you know, is celebrating its twentieth anniversary, and the National Cancer Institute has been located in Bethesda for more than 30 years, but NIEHS has been in existence less than 3 years. Consequently, I will spend some time in giving you a general description of and information about this new Institute and its facilities When the responsibility for developing the Environmental Health Sciences Center in Research Triangle Park, North Carolina, was transferred to NIH in November, 1966, the Division of Environmental Health Sciences was created. In January of this year, its status was changed to Institute, the only one of the NIH Institutes that is not located in Bethesda

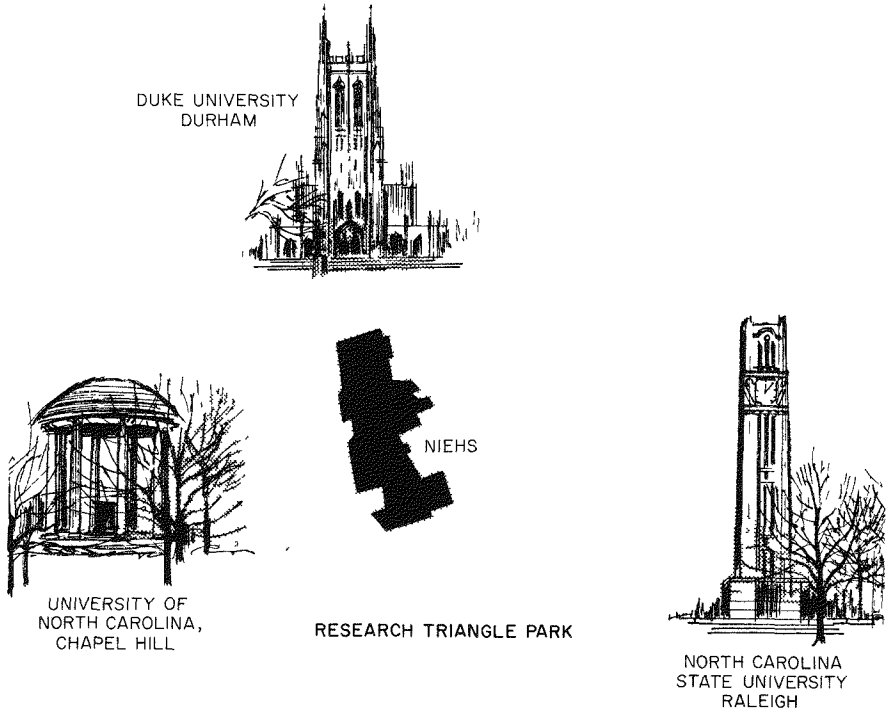

Fig. 1 – Schematic drawing of the Research Triangle, showing the location of NIEHS in relation to the three universities nearby.

I would like for you to see a sketch of the Research Triangle (Fig. 1), not just so you will be able to find us but because the location of the Institute has a definite influence on our relationship with other organizations in the area. It is located within the triangle formed by Raleigh, Durham, and Chapel Hill; these cities are the locations of North Carolina State University, Duke University, and the University of North Carolina at Chapel Hill, respectively. Our relationship with the universities is important not only because resources such as libraries are available, but also, and more significantly, because our professional staff can be affiliated with a teaching institution. There will be increasing opportunities for our staff to do cooperative research with these institutions, to participate in training activities, and to have graduate students work at our facilities under the guidance of our scientists.

The facilities that we are occupying at present (Fig. 2) have been leased from the Research Triangle Foundation, but we are planning permanent facilities to be located on an adjacent 509-acre tract which will be shared with the National Air Pollution Control Administration.

The mission of the National Institute of Environmental Health Sciences is, first, to determine the magnitude and significance of the hazard to man's health

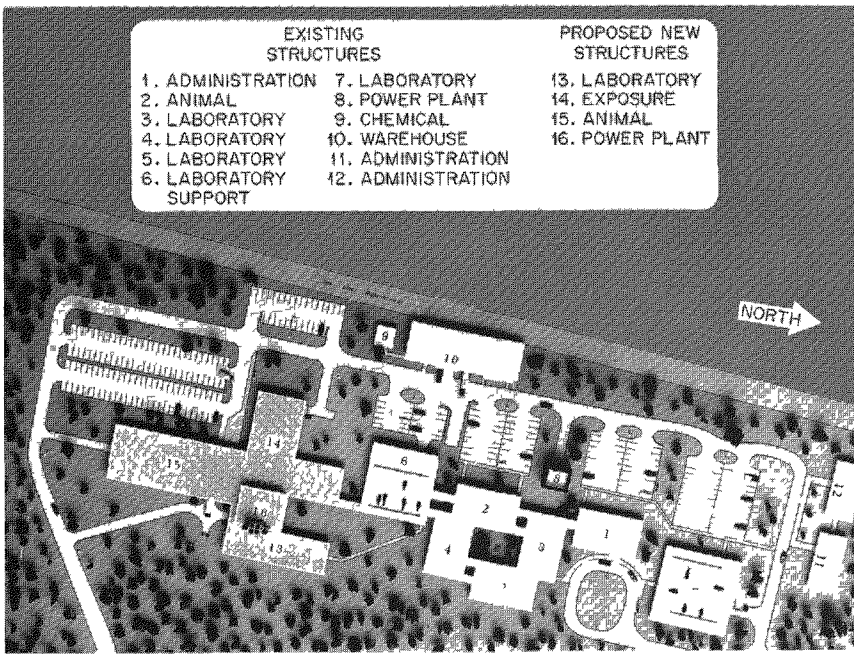

Fig. 2 – A model of the interim facilities of the National Institute of Environmental Health Sciences at the Research Triangle.

of long-term exposures to low-level concentrations of agents – chemical, physical, biological – regardless of their route to man; and, second, to identify underlying mechanisms of adverse response, with the hope that principles related to the response can be established. These principles can then serve as a scientific basis for criteria on which to set standards and can provide predictive guides to be used by control agencies for protective or preventive measures.

As an Institute, this organization makes use of many mechanisms for carrying out its mission – grants, fellowships, contracts, and intramural research including epidemiology and biometry as well as laboratory studies. Our program is being developed on the premise that a structured and coordinated program – incorporating laboratory experimental data, clinical studies, and epidemiological observations – affords the best opportunity for determining the pathogenesis of adverse effects as well as relating the exposure to morbidity and mortality. Instead of developing clinical facilities, we plan to work cooperatively with the two medical schools in our area, using their studies of patients to acquire data that cannot be obtained by laboratory or epidemiological studies.

We are developing an epidemiology and biometry program to help bridge the gap between laboratory and man. We are, for example, cooperating with the Bureau of Occupational Safety and Health in the study of uranium miners, not to determine whether a hazard exists for these workers – that has already been

established – but to obtain information that will be useful in determining the permissible level of exposure to radon and its decay products in the mines. In another study, the effects of exposure to emissions from coke ovens in steel manufacturing plants are being determined. This study will not only help define the hazard to these workers but will afford an opportunity to determine whether organic compounds that are laboratory carcinogens are indeed carcinogenic to man. Epidemiology also provides a means of evaluating the effectiveness of control measures and standards.

In the laboratory the full range of medical, biological, and physical sciences is utilized in studying the effects of environmental agents in small animals and in various *in vivo* and *in vitro* systems.

Again, I would like to refer to our facilities because of their importance in the development of our research program. In Fig. 2 the complex of three buildings on the left represents our proposed expansion of the interim facilities. This complex will include badly needed additional animal facilities as well as general laboratories, but of more interest to the present discussion is the building that will provide aerosol-toxicology facilities. There will be a large area for exposure chambers of various types, as well as biological and chemical laboratories for support of the work that is carried on in the exposure chambers. The incorporation of inhalation or whole-body exposures into our studies is essential not only because the respiratory route is an effective means of administering an agent, but also because it is a common route of exposure for man. Through inhalation or whole-body exposure, we can determine:

1. The stability and persistence of an agent in the environment.
2. The existence of a threshold limit.
3. The specific nature of the effect and the response of the host to the agent.
4. The effect of multiple exposures on the induction of disease as well as the varying response as influenced by the state of health of the host.

Investigation of the rapidly increasing number of agents in the environment is formidable, but meaningful information can be obtained only by multifactorial analysis of studies at realistic concentrations of combinations of chemical, physical, and microbiological agents and host factors.

DISCUSSION

C. G. Baker: Thank you, Dr. Payne. I think the Organizing Committee was experimenting when they put this session on at the end of the regular program. Oftentimes an excellent symposium-type scientific conference is held, the state of the art is reviewed, everybody learns a lot and has a good time, but then the subject is left dangling and one wonders whether any subsequent action will ever result from the meeting. In spite of the great complexity of the subject and the short time available for presentation, we have nevertheless attempted to portray

the possibilities for further action, with presentations of program plans from three major agencies concerned with inhalation carcinogenesis. There are so many complex factors involved in this difficult area of formulating sizeable research programs, of implementing programs at many different places at once, of coordinating programs, and of determining the role of the Government staff in the agencies, that we have done no more than to begin to bring out some of the complicated issues.

It may be disturbing to some, in times when we have no increases in budgets, to talk about plans that extend far beyond current operational capability. I would say that it is important to have plans well beyond current capabilities, since this provides a means for better priority selection and for being ready to respond to questions as to whether additional opportunities for expanded research exist. Occasionally the NCI staff is asked what could be done if more money were made available for cancer research. Dr. Endicott had this question asked of him at the most recent House Hearings, and it is useful to be able to demonstrate that fields are ready for expansion, that the opportunities are there. Then the final decision of how much money is allocated for an area such as cancer research is really a political question to be determined primarily in Congress, with many other influences impinging on the decision.

So in a sense it is up to us to make the best case we can in that arena; it does help to have well-developed plans way beyond what is currently going on.

With that comment I will open the subject for discussion.

C. R. Jensen: I am Carl Jensen, with the Bureau of Occupational Safety and Health. We in Salt Lake City are responsible for the epidemiological studies of lung cancer in uranium miners. We are carrying on the studies in close cooperation with the National Cancer Institute and the National Institute of Environmental Health Sciences.

We have the responsibility for an updating of the 1967 mortality analysis done by Drs. Lundin, Archer, and Holaday. This updating will not be very extensive. The 1967 analysis brought the exposure levels up to 1963, and stopped there; so in our updating we will bring the working-level-month exposures up to 1968. In addition, we will try to improve this analysis on the basis of improved work histories.

Second, we are developing three more cohort groups. The original group, on which the mortality analyses have been made, consisted of about 4000 miners who were identified during physical examinations made by a Public Health Service team in 1954, 1957, and 1960. The next cohort group will be Uranium II. It will consist of all uranium miners who were not picked up in the original cohort, Uranium I. The dimensions of Uranium II are really not set. We don't know how many miners will be in the group at this time – probably 10,000 or so.

We have another cohort group, the Hard-Rock Cohort, which will consist of about 12,000 to 13,000 miners who worked in hard-rock, not uranium, mines

and who were identified by physical examinations made by the U.S. Public Health Service from 1958 to 1960.

The final cohort group will be the Potash Group. It consists of about 10,000 to 15,000 miners who have worked in potash mines in southeastern New Mexico.

The difference between these groups will be a matter of levels of exposure to radon daughters. Unfortunately, the cohorts are not ideal, for other environmental variations exist. But the original group, Uranium I, had an average exposure of, let us say, something near 10 working levels [WL]. We would estimate that the Uranium II group had an average exposure of around 3 to 5 WL – this is atmospheric levels of exposure – and that the Hard-Rock Group probably had an average exposure of no more than 0.3 WL.

There are hard-rock mines that do have higher levels of exposure than 0.3 WL, but they are red-tagged.

Finally, the miners in the Potash Group have been subjected to very low-level exposure. The potash mines are in an old sea bed, shielded from the bedrock by salt. Here the level of radon daughters is less than 0.1 WL. The working environments of all cohorts are mining environments, so they have much similarity; unfortunately, there are dissimilarities which we will have to recognize.

Baker: Thank you, Dr. Jensen. I am sure we will all look forward to seeing these results, since they again offer a chance to tie together animal experiments and the human situation.

G. A. Poda: First of all I'd like to say that I've learned an awful lot here today. I'm not an experimenter; I'm a clinician. And the type of material presented at this conference is what we in the field of prevention and treatment don't often get exposed to.

I would like to amplify what I've said by giving some of the practicalities that we need but that we are not getting. A large number of our experiments involve particle size; we are very concerned with determining the exact particle size. Yet, practically, when we get an exposure of plutonium dioxide dust, or something similar, we never know the particle size. It could be from part of a micron to 50 microns. I would hope that we can get somebody interested in doing the practical inhalation-type experimentation that would give us some assistance with this type of problem.

Also, I'm wondering if some of the agencies represented here today might not be able to summarize the salient points that the people in the treatment field might find instrumental in helping solve their problems.

Baker: I'm going to ask Dr. Richmond to respond to that one.

C. R. Richmond: I agree with you whole-heartedly. When I hear someone ask about particle size, I'm always curious as to what good the information will be to the person if it were available. But your observation is very accurate. If one

tries to get an estimate of particle size for those cases where there have been exposures, one finds variations depending on the position in and around the dry box, or whatever environment under which the accident occurred.

Now, what I didn't mention in my talk was that according to first principles, you try to prevent contamination. However, after it occurs, you then have to assess what has happened. There is a rather large program that the AEC is now supporting which is strictly designed to detect lung burdens, or body burdens, for inhaled materials. I said nothing about this program, but there is a whole variety of detection systems being developed – for example: proportional counters, solid-state detectors, and sophisticated dual-scintillator-type detectors, which are now capable of detecting fractions of a body burden of ^{238}Pu or ^{239}Pu.

Once the material is in the body – to be more specific for your problem, in the lung – then if you have an estimate of particle size you may be able to make a somewhat more educated guess about early clearance. Usually it is too late by the time an accident is recognized to really worry about early clearances. I think the problem, once an accident occurs, is: What can you do about it? And that is the last part of the program I mentioned earlier: finding possible means of reducing a body burden or accelerating the loss of the acquired burden.

But I agree with you and I see your problem. It is very difficult to get biological information on a whole host of radionuclides. It is virtually impossible because of logistics and numbers of animals. The emphasis I put on particle size was specifically to answer this problem. That is, can we make any generalizations about the effects of particle size and number. This is, if you will, the pure radiobiology aspect of the problem. For reasons of practicality, it won't help you.

C. Kensler: Carl, in your talk you indicated that you were somewhat enamoured with the modern management systems approach to tackling the major, broad-scale problems in inhalation carcinogenesis, and Dr. Saffiotti indicated the use of this approach at an institute level, as did Dr. Richmond. But I detect in your comments a note that the interagency planning was going to be left on a very informal basis, and I wonder if there wouldn't be a place and a need at this time to have some subdeputies from the three agencies try to plan a systems approach that could be implemented when funds became available, so that the politics of where to put it could be avoided. Do you have any plans for interagency program planning in a systems manner?

Baker: I think the simple answer is no, as of the moment. By and large there exists an informal kind of an arrangement for keeping track of related activities, partly through a series of interlocking meetings. Because of frequency of these meetings, attended by senior staff of the different agencies, issues are continually covered that cross these organizational lines. We are therefore probably a little more informed of each other's activities than it might appear.

On the other hand, I do not want to suggest that plans are systematically being developed in a tripartite, single system; they are not.

I might admit straightaway that there are some people who would think we should not be planning by the systems approach even in *one* agency, much less in *three!* My answer to that is that I believe we need both the systems approach for seriously attacking certain kinds of problems and the pluralistic, loosely or noncoordinated approach for other areas, especially in dealing with long-range less well-defined objectives.

H. G. Boren: Dr. Baker, all three of the agencies have outlined very well the systems approach. We have also talked a lot about the design of experiments. But I would think that unless we develop more effective ways of continuing our communication between people with different interests, we are due to failure.

I think this meeting is a good example of trying to do this. Here we see nice support, and yet we say that it will be six months before we have a publication. I seriously doubt if our present method of publications and meetings is an effective enough way of communicating what we are doing, and I would make a plea that we must have communication between all of our systems approaches, and would hope that we will seriously explore new ways of doing this.

Baker: I certainly would agree with that. Sometimes I wonder if the mania for publishing papers has not really gotten out of hand. Moreover, as a means of communicating certain kinds of information it is antiquated and inefficient. The problems that arise when other approaches are tried, however, are rather tricky to handle. When the systems approach is well done, it includes coverage of information handling. Indeed, to monitor program effectiveness, information requirements must be detailed, including specifications in experimental design, format, flow, and use of the information at receiving points. This subject is another topic suitable for a working conference, though I failed to mention it in my summary. It is going to take some of the best minds we have to solve this problem.

U. Saffiotti: I think that this problem in communication is indeed very urgent. We at the National Cancer Institute have been giving consideration to ways for improving the current situation. One way that has been used in other parts of the Etiology Program, particularly in the Viral Oncology Program, is to subdivide the total effort into segments, centered on the major problem areas, and then have frequent discussion meetings of people working in a joint program on related issues. We are in the process of trying to develop such a segmentation of our total effort in chemical carcinogenesis, so that at least we will have areas of activity where the interrelation will be easier between groups in our Institute and in the collaborative programs.

W. W. Payne: I would just like to add that we have used, in several instances, this mechanism of working conferences to get people together to consider a specific problem. The subject of one of them has been referred to several times

in this meeting, namely, the uranium miner problem and radiation hazards in general. We arranged a small meeting in which the various organizations that were interested in that problem, and were working in it, were represented. I think it was very fruitful in determining the portions of the problem that are not being pursued.

Baker: We have had considerable success in bringing together, at annual meetings, the many investigators who participate in the Viral Oncology Program. Just as NASA built a real esprit de corps in the Moon Shot effort, Dr. Frank Rauscher, Program Director, is developing a similar motivation and joint effort in part through these annual meetings, which update the status of the program around a delineated theme. During this updating, consideration is given to possible changes in main objectives and direction, the progress of the program is reviewed, and its immediate projection is stated. It takes a good bit of effort, of course, to effectively plan these kinds of meetings. For this year's meeting, Dr. Rauscher has a whole series of very key questions that he hopes to pose to the participants so that their views can be used for possible alterations of the program. Some people are fearful that plans such as these will become straightjackets. They can, but it is essential to keep working to update them.

Aside from the Viral Oncology Program, I would say that the Chemotherapy Program is furthest along in this kind of approach, that is, in the detailed laying out of objectives and key decisions that must be made, in delineating the criteria for allowing one to make the decisions, and in specifying the kinds of data that must be generated to make them. The plans then form a "yardstick" against which the successes of generating and using the data for making necessary key decisions can be measured.

When this kind of specification is done, it becomes easier to answer some of the questions of communication across a broad front. To formulate well the appropriate kinds of questions and to specify the required types of data is a very difficult job, but I think we are beginning to learn how to do some of this. Whether we are going to have the resources to implement these large-scale programs is the issue. To solve the problems in carcinogenesis will require large, multidisciplinary, integrated efforts. Frankly, I am not optimistic that the necessary investment will be made soon.

J. F. Park: If I interpret correctly, the major progress of cancer research during the past few years toward reducing the number of people who die from cancer has been in early detection and early therapy. Are there any special programs which, by use of some of the animal models, are designed specifically for the purpose of increasing our ability to detect pulmonary neoplasia at an earlier stage than what we now recognize as frank neoplasia? In the studies reported at this conference there are many indications that there are cellular changes leading to cancer. We talk about precancerous lesions, but is there an effort to isolate particular cellular responses, and say: This is an early indication

which now, at this point, might be more susceptible to chemotherapeutic treatment than once neoplasia has progressed to the stage that more heroic attempts at therapy are required for treatment?

Baker: Let me comment a bit about some history first. About 15 years ago the National Cancer Institute embarked on a program of evaluating the various tests and diagnostic procedures claimed to be of aid in early diagnosis and detection. These were by and large morphological approaches (cell observations) and/or serum studies (serology or chemical studies, e.g., enzymology and the like). With the help of a number of institutions, something over a hundred of these various procedures were evaluated with sufficient statistics to allow valid conclusions: with the exception of the Papanicolaou smears for exfoliative cytology in surgery, none of the procedures were any good.

This led, I think, to some dampening of interest in diagnostic aspects of cancer research. Many people still feel that the leads in this direction are not good. I am speaking now of the research end of it, rather than the application end. However, in the special 1969 appropriation of $1,200,000 for lung cancer work, $200,000 was allocated for diagnostic work aimed at earlier detection of lung cancer.

The programs that have been concerned primarily with applying findings for diagnosis and detection to human populations have been the Cancer Control Program, which is not within NIH but is a different part of the Public Health Service. I understand these programs are perhaps going to disappear because of budget activities, although some of the functions presumably will be maintained through other programs, such as the Regional Medical Program and similar activities.

As regards mammography in the breast cancer field, we have made a fair investment in trying to find out if the mortality is going to be changed. The question of whether earlier diagnosis does, in fact, lead to an improved mortality situation is not well demonstrated in very many forms of cancer. It seems likely that reduction in cervical cancer mortality over the last few years is in part due to earlier therapy, brought on by greater use of the Papanicolaou smear. The proof of this does not appear to be established, however. Others may want to argue the other way.

In conclusion I would again like to thank everyone participating, especially the ORNL staff. I am reminded of Dr. Kleinerman's remark after he had gone through a number of rather complicated studies and slides; he said: "At any rate, let's get on with it." I charge you to go forth and get on with solving carcinogenesis problems.

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