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PACIFIC NORTHWEST LABORATORY
ANNUAL REPORT FOR 1971
TO THE
USAEC DIVISION OF BIOLOGY AND
MEDICINE

VOLUME I LIFE SCIENCES
PART I BIOLOGICAL SCIENCES

SEPTEMBER 1972

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PACIFIC NORTHWEST LABORATORY
ANNUAL REPORT FOR 1971
TO THE
USAEC DIVISION OF BIOLOGY AND MEDICINE
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PART 1 BIOLOGICAL SCIENCES

by

The Staff of the
Biology Department

Edited by
R. C. Thompson

September 1972

BATTELLE
PACIFIC NORTHWEST LABORATORIES
RICHLAND, WASHINGTON 99352

This is the 21st Annual Report of the Biology Department. Previous volumes are: HW-25021 (1951), HW-28636 (1952), HW-30437 (1953), HW-35917 (1954), HW-41500 (1955), HW-47500 (1956), HW-53500 (1957), HW-59500 (1958), HW-65500 (1959), HW-69500 (1960), HW-72500 (1961), HW-76000 (1962), HW-80500 (1963), BNWL-122 (1964), BNWL-280 (1965), BNWL-480 (1966), BNWL-714 (1967), BNWL-1050 (1968), BNWL-1306 (1969), and BNWL-1550 (1970).

Part 2 of this volume, BNWL-1650 Part 2, covers work in the ecological sciences. Volume II of this report covers work in the physical sciences and is issued in three parts: Atmospheric Sciences BNWL-1651, Part 1; Radiological Sciences, BNWL-1651, Part 2; and Instrumentation, BNWL-1651, Part 3.

FOREWORD

With this twenty-first Annual Report from the Biology Department, the series may be said to have come-of-age. If coming-of-age implies the attainment of a certain maturity of style or presentation, the analogy is hardly appropriate. If, however, it involves some sense of transition, the analogy is more apt.

The present Annual Report clearly suffers from a kind of "identity crisis." It contains many reports in the abbreviated style typical of recent past Annual Reports and many others that are much longer and more detailed. This move toward more detailed reporting is at the request of our investigators. They balk at reporting their results in a form so short on detail as to be, from their point of view, scientifically valueless. We hope that this revolt of our investigators will not occasion a counterrevolution on the part of our readers. We would appreciate receiving your comments on this probably irresolvable problem. Do you welcome the additional detail in the reports of special interest to you or is the total mass of paper simply too formidable?

Because of the greater effort required in producing the lengthier reports and because of our firm resolve to issue this Annual Report within six months of the close of the reporting period, it has been necessary to go to press with a few program areas not covered. These will be covered in next year's Annual Report which it is our firm resolve to issue within three months of year's end.

Individual reports, as in the past, are grouped according to AEC budget categories. Following the reports is a complete listing of the Department staff, as organized at year's end. Early in 1971 there was an extensive administrative reorganization of the Department with the personnel of seven Sections reallocated to four. This move had relatively little effect on Department operations except with regard to the flow of paper.

Our new Life Sciences Laboratory was dedicated by Dr. John R. Totter, Director of AEC's Division of Biology and Medicine, on September 27, 1971. Dr. Totter's remarks on that occasion are included in this Annual Report. Also included are the dedicatory remarks of Dr. H. M. Parker, former manager of Hanford Laboratories, and Dr. H. A. Kornberg, former manager of the Biology Department.

The dedication of our new laboratory was a feature of the Eleventh Annual Hanford Symposium on the Biological Implications of the Transuranium Elements held from September 27-29, 1971, under the joint chairmanship of Drs. W. J. Bair and R. C. Thompson. The Proceedings of this symposium, including some sixty

papers, will appear as the June, 1972, issue of Health Physics. The Twelfth Annual Hanford Symposium on Radionuclide Carcinogenesis is scheduled for May 11-13, 1972.

A list of 1971 publications by personnel of the Biology Department appears at the end of this Annual Report. Requests for reprints will be honored as long as the supply lasts.

W. J. Bair, Manager
Biology Department

REMARKS AT THE DEDICATION OF THE
LIFE SCIENCES LABORATORY

BY

DR. JOHN R. TOTTER, DIRECTOR,
DIVISION OF BIOLOGY AND MEDICINE

It is no small responsibility to give the dedication address at these ceremonies for the new Life Sciences Laboratory. I feel it is appropriate today that I start my comments by briefly reviewing some of the outstanding research which has been conducted here at Hanford.

This may be a good time to invoke a bit of philosophy that is germane to my remarks. The philosopher, George Santayana, once said, "Those who cannot remember the past are condemned to repeat it." While it is of paramount importance in science to look ahead, it is often helpful to see where we have been, to evaluate what we think we know and to appreciate those things we don't know. I should like to continue in this retrospective-prospective tradition by directing my remarks towards both the past and the future.

As some of you in this audience know first hand, the Hanford Project began operation in 1944 with the assignment to supply plutonium for the Manhattan Project. Along with the awesome production reactors and chemical separations plants, there developed a fledgling monitoring and biological research project. It appears that the Biology Lab got its start when Herb Parker hired Harry Kornberg around 1947 to look into the possibility of identifying biochemical changes in blood that might be produced by exposure to radiation. Parker has been at Hanford since the start of operations as, I believe, Head of the Health Instruments Group. Incidentally, some interesting radioecology work, although probably not known as such at the time, was in progress in the late 1940's when Parker and colleagues monitored rabbit thyroid glands for ^{131}I

During these early years, work was done on the oral uptake of ^{238}Pu and ^{239}Pu . Of historical interest is the fact that the ^{238}Pu was obtained from Berkeley, via Dr. Seaborg, and followed by only a few years the discovery of plutonium in 1941. Other studies were undertaken by Dick Foster, who had been working at the University of Washington's Fisheries Lab under Lauren Donaldson, to determine the possible effects of reactor effluent on fish.

I mentioned earlier the importance of occasionally looking back as we move ahead in science. This reminds me that things have a way of being rediscovered periodically; sometimes this must be pointed out

to those who cannot remember the past. I am certain that those who have only recently discovered such things as reactor effluents - and tritium - could profit a great deal by looking at the development of the life sciences program here at Hanford.

In late 1945 and early 1946, trout were placed in troughs over which passed different concentrations of reactor effluent water. I wish I had time to develop for you the many interesting studies which have followed through the years in this area. Project ALE (Arid Lands Ecology) and the aquatic ecology studies conducted within the Ecosystems Department are evidence that serious thought has been given to future activities.

Around 1948, Kornberg and Parker formed a Biology Group and proposed a program which was presented the following year to the AEC's Advisory Committee for Biology and Medicine. This was indeed prospective in scope and probably marked the transition from the earlier emphasis on monitoring to a broader based concern for radiation effects, metabolism, and the use of radionuclides and radiations as tools in biological research. This was accomplished under the management of the General Electric Company. In 1965, the Battelle Memorial Institute assumed the management of research and development activities at Hanford.

The ACBM agreed with the proposed program and suggested that an existing building be converted to a temporary biology laboratory until a new one could be built. At that time AEC Commissioner Johnson was Manager of the General Electric Company at Hanford. To this end, a surplus building in the 100F Area was chosen - in part because of its proximity to the fish ponds. The biology group moved into the converted building in 1950 and produced an annual report in 1951.

This general period witnessed the organization of a Botany Group which initiated work on the absorption and translocation of radionuclides in plants. During the 1950-55 period, the animal farm additions developed. The AEC recognized the importance of life-time animal studies designed to study the late effects of radiation. One of the earliest, related to chronic exposure to reactor-produced ^{131}I , was initiated at Hanford in 1948 using sheep as the experimental animal. Concern about extrapolating these data to humans led to the development of the Hanford Miniature Pig as a research animal.

Another long-term study, begun in 1958, involved daily feeding of ^{90}Sr to pigs. This program spans three generations of animals - about a thousand - and still continues to provide useful data. Again, looking to the future, this effort has evolved into the exciting area of the leukemogenic process, per se. This is another example of an effort which is both mission oriented and basic in scope.

The early 1950's witnessed animal experimentation under the direction of Roy Thompson and colleagues. It is no surprise that this work was related to the development of the hydrogen weapon. What is a surprise to many scientists is that some people are rediscovering today things that Roy Thompson and coworkers found - and published - about 20 years ago. Some of this work represents the definitive experiments on the radiation biology of tritium in mammals.

In 1959, additional laboratories were added at the animal farm primarily for inhalation studies. Several years later a three-story addition provided new small animal quarters, a ^{60}Co irradiation facility, and the luxury of a conference room and library. The year 1964 marked the loss of the Aquatic Laboratory from fire. In 1967, anticipating the move to the 300 Area, dog and pig facilities were constructed adjacent to the chosen site.

Another example of far-reaching research here at Battelle is the inhalation program. The work on radioactive particles and pulmonary inhalation began in a peculiar fashion. Bill Bair, possibly one of the first Ph.D.'s in radiation biology, had just completed his work at Rochester under Newell Stannard. He came to Hanford to continue his work on yeast. It happened, however, that one of the chemical processing plants began to emit particles of ^{106}Ru ; and in an effort to learn the effects of such particles, Bill Bair was asked to help out in a radioactive particle program. The particles were put on the skin and were fed. They were also instilled into the lung intratracheally, and finally an inhalation system was designed. From this start has grown the Lab's superb inhalation capability and its outstanding program on the transuranic elements.

Although emphasis has been on plutonium, the technology which resulted has a general application to our understanding of pulmonary deposition, retention, and translocation of inhaled particulates, that is, to the broad problem of airborne pollution. One particular study about 12 years old is of special significance as it has provided us with much information on the late effects of inhaled plutonium. This work has led to new studies, using even smaller initial lung depositions of plutonium, which will extend into the future.

Other work here at Battelle is related to uranium mining problems. More important, perhaps, is the fact that this work represents a serious effort to investigate the effects of radiation and other agents which may act additively or synergistically. Many people believe that we shall see much more of this kind of experimentation in the future.

Studies related to the artificial heart, which are now in progress, developed as an outgrowth of the proven competence in research on the implantation of thermal heaters in the thoracic aorta of miniature swine, performed for another agency.

If this quick review of the historical highlights has slighted anyone, I can assure you it was not intentional, except in the sense that it would take much too long to recite all of the significant achievements accomplished here.

I would also like to take this opportunity to discuss some of the more philosophical aspects of scientific research. Today we hear a great deal about the impact of technology on society and the failure of science to solve many contemporary problems. I suppose if I were asked what one factor had contributed more than any other to the so-called disenchantment with science that seems to be occurring, I would say communication. I should like to amplify for just a few moments my thoughts on this subject.

For a long time, it was my strong conviction that if one did good basic research, that would be ample justification for continued federal research support. However, in the past few years I have acquired much experience testifying before various Committees of Congress on the Atomic Energy Commission's biomedical and environmental research program. I think I now realize, as well as anyone here, that one must be prepared to explain to the Congress and to the public the significance of basic research in understandable terms. Just doing good work is not enough. We must be prepared to explain its value in competition with other demands. It is my view that most basic research can be presented to the public and the Congress in terms of broad national needs and goals. For example, if we are going to understand completely the effects of radiation on cells, we must first understand how the cell functions normally. Those of us speaking on behalf of science are doing our best to point out to the Congress both the immediate and long-term value of continued federal support of research. However, neither I nor other responsible federal officials can discuss this need with interested groups in all the local communities.

Just as you accept the opportunity to continue your research in this beautiful new facility we are dedicating here today, I hope you will endeavor at every opportunity to discuss with lay audiences the value of such research. I realize that this raises a special difficulty in terms of news media, newspaper, radio, television since the media may become impatient with the scientists' technical jargon and carefully chosen words. Occasionally, a reporter in the interest of

dramatic effect or readability may render statements in a fashion' **which** to the scientist would be considered unscientific. There are few who speak in public who have not been caught in such a difficulty. Rather than criticize the newsman for his failure to understand science, one might just as honestly criticize the scientist for his failure to make himself understood. The scientist has an obligation to attempt to explain his work to the nonscientist.

The Life Sciences Laboratory which we are dedicating is a multiple discipline facility. Scientists can conduct work ranging from fundamental radiation biology at the cellular and molecular level to **major studies** in large animals from basic terrestrial and aquatic investigations to mathematical modeling of major ecological systems. Many of these studies might well be considered fundamental or basic research, but they are vital to developing a complete understanding of the effects of radiation on man and his environment. I believe such research is a coordinated long-term program which continues to contribute to our knowledge in this important area. We recognize, as I think you do, that each piece of research contributes in its own unique way to our overall understanding.

But something more is needed. It is important that the public understand this need. Such an understanding can, I believe, lead to a broader realization that the use of our natural resources, and here I would include our scientific talent and facilities, have been and are working for the betterment of our society. It has been said that last year's basic research is often this year's applied research. I think whether research is regarded as basic or applied depends on the time frame within which one views it.

Certainly our understanding of the fundamental life processes is still elementary and any advance in understanding is very likely to find applications rather quickly in medicine or in agriculture. Certainly the research which will be conducted in this new facility will have applications far beyond the immediate problems with which we are concerned.

While the results of your work will have direct application to the Atomic Energy Commission programs, some discoveries will also have application in the future in ways we could not foresee at present. It would be presumptuous of me to attempt to predict specific contributions your researches might make to mankind many years in the future.

A strong program in basic and applied research is necessary to the Atomic Energy Commission and, I think, to the nation. I hope I have convinced you to join with me in attempting at every opportunity

to explain in an understandable fashion to the nonscientist why a strong and continuing program is necessary.

In closing, may I wish those of you who will have the opportunity to continue your work in this new facility every success.

Thank you.

REMARKS BY
H. M. PARKER, FORMER DIRECTOR
HANFORD LABORATORIES

Today is a day when all biologists should be looking to the future and what they can accomplish in the fine new Life Sciences Building that the AEC has prepared for them. Perhaps I can be excused for looking backward briefly to the origin of the biology program at Hanford. This program began in 1946 shortly after the General Electric Company took over the operating contract for the Hanford Works. It arose as a direct felt need of the radiation protection group at this location which in those days was known as the Health Instrument Division, commonly abbreviated as HI because "radiation" was a dirty classified word when Hanford started. It was conceived as a practical program in radiobiology to solve the problems that had arisen concerning hazards to man in the plant and the life forms in the environment.

Karl Herde, whom I hoped would be here today, was one of the first biologists to work on this program as was Dick Foster, who is still with us today. (Dick was indeed the first biologist working at Hanford, but originally he was in a separate division and it was only in September 1946 that we were able to seduce him into the HI Division.) In early 1947, I was able to acquire the services of Dr. Harry Kornberg as Manager of the Biochemistry Section of the biology activity. It was not long before Harry persuaded me that he should be manager of the biology program, a position that he filled with distinction for 20 years. Harry developed the original, wholly practical environmental and biology program into a good balance of applied work and enough basic research to keep our senior scientists from going insane, for the most part. Practically every one of his groups developed at least one long continuing program which has become a classic in radiobiology research in the United States. I have mentioned Dick Foster; his work on the Columbia River problem is such an illustration.

For brevity, I will single out only two more. One was that run by Leo **Bustad**, whom I am most happy to see with us today, in the classic program of radioiodine effects on sheep and later on other animals. Let me cite one other program which is still being conducted by the current staff, and that is the internationally recognized Inhalation Toxicology program of Bill Bair.

This country behaves as though it discovered the words "ecology" and "environment" about five years ago. Since that time, the AEC has been clobbered by its critics on all sides because it is said to have done a fairly respectable job of protecting workers in the atomic plants but has paid no attention whatsoever to ecology and the problems of radiation in the environment. The program that has been operating at Hanford for twenty years is a complete refutation of this which deserves to be better known. The principal programs that were started twenty years ago and have been maintained to the present time were in fact mainly concerned with the environmental problems.

Harry, will you please join me at the podium, and the two senior resident managers, Dr. Bill Bair of Biology and Dr. Burt Vaughan of Ecosystems, will you please join us? In recognition of this day, Harry and I have jointly drawn up plaques to wish you well in the new laboratory and to commemorate the origins in the HI Division. This plaque is based on the logo used by HI in the early days. The logo that you see, if you can see from the audience, represents a shield of radiation protection for people and the environment. HI is in the center and it is divided into three sections which represent, in the upper left, the biology program symbolized by the microscope. On the right hand side, the bio-physics program, symbolized rather insultingly by a voltmeter and below it, personnel monitoring, represented by the old CUTIE PIE instrument. It will be of interest to the biologists here to recall that it was actually Jerry Davis, who was a senior member of Dick Foster's team at that time, who drew this logo. That is why biology appears in the key position in the first place on the left. However, those of you familiar with British heraldry will also remember that the left hand side of the shield is the sinister side. Harry and I have great pleasure in presenting these plaques jointly to you, Bill and Burt; and we wish you the best in your programs in this building.

REMARKS BY
H. A. KORNBERG, FORMER MANAGER
BIOLOGY DEPARTMENT

As this building today is dedicated to research in the life sciences, we should recall that part of it serves to complement a physically much larger project--the Arid Lands Ecology Project. The 120 square miles along the northeast slope of Rattlesnake Mountain is one of this country's major ecology reserves, and the man who did most to bring it into being and into perpetuity is the previous speaker, Herb Parker. It was my privilege to have worked for and with him during the 20 years that life sciences research grew from a minor effort in biochemistry to one of this Nation's major laboratories in biology.

It is rare that a scientist has the opportunity and distinction to lead the building of a laboratory. Today I become one of the very few who have had that distinction twice. Herb Parker recalled some trials and successes during the 20 years when that occurred. A chapter he omitted, with no reluctance, I'm sure, concerns alligators. Eight years ago we were using them in our experimental program. We observed that alligators have two distinctive characteristics: they hiss at managers and laboratory directors and are great escape artists. To commemorate a year made especially eventful by those characteristics, one of the alligators was stuffed, mounted, and presented to me at Biology's 1963 Christmas party. This handsome little fellow has been my constant office companion ever since. And since we are here to dedicate a great new laboratory, I would like, in turn, to commemorate that event by bequeathing my little friend to the laboratory building. It is my pleasure to present him to the Building, care of its manager, Mr. Ray Hultman.

You who made this laboratory possible have my fondest wishes for continued success and for the satisfaction that achievement brings.

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GASTROINTESTINAL IRRADIATION STUDIES

This long-continuing project has included studies on the physiologic and pathologic changes in the intestine of small and large laboratory animals exposed to external radiation sources. Work in this general area during 1971 was curtailed due to a shortage of personnel and a diversion of effort to studies with californium-252 as a radiation therapy source. This work is described in the section, "Miniature Swine as Model for Intracavitary Irradiation."

We expect to continue our studies of the functional effects of irradiation of the gastrointestinal tract, employing neutrons from a ^{252}Cf source; and to expand our investigations of various compounds of transuranic elements, as represented in this Annual Report by studies on the absorption of plutonium chelates.

FACTORS AFFECTING PLASMA PROTEIN LEAKAGE
INTO THE IRRADIATED INTESTINE

Investigators:

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Technical Assistance:

Alma L. Crosby

By means of whole-body autoradiography, it was demonstrated that 3 days after 1000 R of X-irradiation plasma protein pooled in the intestine. Leakage into the lumen of the intestine was decreased by feeding a sodium deficient diet or by pre-treatment with the radiation protective agent WR-2721.

Previous studies of gastrointestinal tract radiation injury reported from this laboratory (Annual Report, 1969) demonstrated that injected ^{131}I -labeled polyvinylpyrrolidone (PVP), and protein labeled by $^{51}\text{CrCl}_3$ injection, was lost into the gut lumen of irradiated animals more rapidly than was the case with nonirradiated animals. This effect was lessened by pre-treatment with the radiation protective agents AET and cysteine (Annual Report, 1965). We showed most recently (Annual Report, 1970) that ^{51}Cr moved out of the blood of irradiated rats, mice, and pigs more rapidly than from the blood of non-irradiated controls. The amount of ^{51}Cr appearing in the gut lumen a week after exposure exceeded the activity remaining in the blood, suggesting an intermediate site as the

source of protein reaching the lumen. We also noted that rats drinking isotonic saline lost more protein than those drinking water.

OBSERVATIONS

To further study the mechanism of protein loss by irradiated rats, ^{125}I -albumin was injected intravenously 3 days after lower body exposure to 1000 R X-radiation. Animals were bled at intervals to follow the disappearance of the labeled albumin from the vascular system. These results, shown in Figure 1, indicate no difference between irradiated rats and unirradiated controls until 3 days after injection (5 days after exposure). The approximate half-time for the disappearance of ^{125}I from the blood of control rats was 1.0 day and for irradiated rats (1000 R) 1.3 days. The behavior of ^{125}I -albumin in the blood contrasts sharply with that of $^{51}\text{CrCl}_3$ (Annual Report, 1970). The

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FIGURE 1. Disappearance of ^{125}I -Albumin from the Blood of Irradiated (1000 R lower-body exposure) and Unirradiated Rats (Six animals per group were injected 3 days postexposure with 10 μCi ^{125}I -albumin. Plotted points are mean value $\pm$ S.E. of mean.)

half-time for disappearance of ^{51}Cr in controls was 1.9 days; whereas, after lower-body exposure to 1000 R it was 2.8 days, indicating that not only radiation, but also the labeling of the protein was affecting the rate at which protein left the blood.

Other rats, injected with 10 μCi ^{125}I -albumin at 3 days after 1000 R, were killed, frozen, and sectioned for gross autoradiography. The autoradiogram, shown in Figure 2, demonstrates a greater accumulation of radioactivity in the intestine of the irradiated rat than was present in the unirradiated control. This suggests that a marked vascular permeability, as well as vasodilation, occurs in the enteric blood supply 3 days after 1000 R.

To study the influence of sodium on plasma protein leakage, rats were depleted of that electrolyte by feeding them a low sodium diet that

FIGURE 2. Autoradiograms of Rats Injected with 10 μCi ^{125}I -Albumin (Left-hand pair, unirradiated controls; center pair, 3 days after 1000 R to lower body; right-hand pair, 3 days after 1500 R to lower body; the upper section in each case is from the left side and the lower section from the right side. All autoradiograms were exposed for 20 days. Note the increased concentration of ^{125}I in bone, kidney, and small intestine of irradiated animals.)

contained 2% Katonium*, a cation exchange resin that binds sodium during passage through the alimentary tract. After 2 weeks on this diet, the animals were lower-body irradiated with 1000 R and injected 2 days later with 5 μ Ci of $^{51}\text{CrCl}_3$ to label plasma protein. Controls were fed a stock diet. Others were given 2% NaCl in their drinking water to produce a high sodium intake. Excreta collections were made daily for 4 days. The results are shown in Table 1. They clearly demonstrate that the sodium intake did not affect ^{51}Cr excretion in nonirradiated rats but did affect losses in irradiated rats. Irradiated animals on a low sodium diet excreted less than half as much ^{51}Cr as rats on a stock diet or on a high sodium diet.

Protection of the gastrointestinal tract by prophylactic treatment with cysteine or aminoethylisothiuronium bromide hydrobromide (AET) results in a reduced excretion of ^{131}I -PVP and, presumably, plasma protein. The protective agent S-2-(3-aminopropylamino) ethyl phosphorothioic acid hydrate (WR-2721) was evaluated for its protective effect on the bowel by injecting it intraperitoneally at 1 hr pre-exposure. The results shown in Table 2 indicate some protection against 1000 R, as evidenced by a decreased excretion of ^{51}Cr , but no evidence of protection against 1500 R. The degree of protection might have been increased if a drug dose larger

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TABLE 1. Effect of Sodium Intake on Excretion of ^{51}Cr in the Feces of X-Irradiated Rats Injected at 2 Days Postexposure

Treatment	^{51}Cr Fecal Excretion(a) (% of administered dose)
Stock Diet Nonirradiated Controls	5.7 $\pm$ 1.7 (6)
Low Sodium Intake Nonirradiated Controls	5.0 $\pm$ 0.4 (6)
High Sodium Intake Nonirradiated Controls	7.4 $\pm$ 0.4 (6)
Stock Diet Lower-body Exposure to 1000 R	17.5 $\pm$ 2.6 (6)
Low Sodium Intake Lower-body Exposure to 1000 R	8.4 $\pm$ 1.1 (6)
High Sodium Intake Lower-body Exposure to 1000 R	15.3 $\pm$ 1.9 (7)

a. Four-day cumulative fecal excretion commencing at 3 days after exposure. Mean values $\pm$ S.E. of mean. Number of rats shown in parenthesis.

TABLE 2. Effect of the Protective Agent WR-2721 on Fecal Excretion of ^{51}Cr by the Rat

Treatment	^{51}Cr Fecal Excretion(a) (% of administered dose)
Unirradiated and Unprotected Controls	5.4 (8)
WR-2721 (300 mg/kg)	4.4 (5)
Lower-body X-ray (1000 R)	19.2 (6)
Lower-body X-ray (1000 R) + WR-2721 (300 mg/kg)	12.1 (5)
Lower-body X-ray (1500 R)	17.1 (4)
Lower-body X-ray (1500 R) + WR-2721 (300 mg/kg)	16.5 (6)

a. Four-day cumulative fecal excretion commencing at 3 days after exposure. Mean values, number of rats in parenthesis.

than 300 mg/kg could have been tolerated, but its toxicity to rats precluded higher dosing. Diarrhea following 1500 R interfered with fecal collections from both the protected and unprotected animals. There was other evidence for protection against 1500 R, however, since all animals pretreated with WR-2721 survived the study, whereas all unprotected rats exposed to 1500 R died before the excreta collection period was completed. A few animals killed for histopatho-

logic evaluation of intestinal damage also showed evidence of protection.

CONCLUSIONS

Irradiation of the rat gastrointestinal tract results in a marked accumulation of plasma protein in the mucosa but the means by which it leaks into the lumen remains unknown. Leakage is, however, sodium-dependent, since a deficiency in that electrolyte resulted in a decreased loss into the feces.

GASTROINTESTINAL ABSORPTION AND RETENTION OF PLUTONIUM CHELATES

Investigators:

D. W. Baxter and M. F. Sullivan*

Technical Assistance:

Alma L. Crosby

The intestinal absorption of plutonium nitrate is increased approximately 700-fold when chelated with DTPA. Within 2 days, virtually all of the absorbed plutonium-DTPA complex is excreted in the urine. Thus, plutonium retention in the liver and skeleton is quite low but more than twice as high as when DTPA is not present. Citrate is less effective than DTPA in increasing absorption but more effective in increasing retention. Since both absorption and retention are increased by chelation, consideration must be given to this added risk when therapeutic procedures result in the chelation of plutonium entering the GI tract.

Intestinal absorption of plutonium is so low that it may be largely ignored as a practical hazard even after ingestion of massive doses.⁽¹⁻⁴⁾

Because of this, the quantity of inhaled plutonium that enters the gastrointestinal tract has been given little consideration, even though the major fraction of that inhaled may be

swallowed^(5,6). Recently, Nenot, et al., have recommended that, "in

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case of respiratory exposure to plutonium, americium, or curium solutions, DIPA should be given as aerosol immediately after exposure." This raises the question of whether DIPA inhalation therapy might increase gastrointestinal absorption of plutonium which is swallowed after leaving the lungs. Plutonium citrate is known to be more readily absorbed than plutonium nitrate.⁽²⁾ The effect of DIPA chelation on the gastrointestinal absorption of plutonium has not been studied.

OBSERVATIONS

Twenty, female, albino rats, average weight 318 g, were divided into four groups. The first group received

plutonium nitrate solution by gavage. The second group received plutonium in a 10% DIPA solution. The third group received plutonium in 4.5% sodium citrate solution. The gavage solution for the fourth group contained both 10% DIPA and 4.5% sodium citrate. The gavage solutions were prepared at pH less than two, and either one or two milliliters were administered. The plutonium dose was 170 μ Ci in each case.

Rats were individually housed in metabolism cages and urine and feces were collected for 5 days. They were then sacrificed with chloroform and the liver and one femur analyzed for plutonium.⁽⁷⁾

Results are summarized in Table 1. Plutonium retention in liver and femur

TABLE 1. Percent of Intragastrically Administered Plutonium (170 μ Ci) Retained in Liver and Femur After 5 Days and Excreted in Urine During the First 2 Days Postadministration (Values are mean $\pm$ standard deviation.)

	Plutonium Administered as:			
	Nitrate	DIPA Chelate	Citrate	Citrate + DIPA
Liver ($\% \times 10^3$)	0.21 $\pm$ 0.09	1.3 $\pm$ 0.5	18 $\pm$ 19	3.1 $\pm$ 1.6
Femur ($\% \times 10^3$)	0.04 $\pm$ 0.02	0.07 $\pm$ 0.03	3.2 $\pm$ 2.6	0.21 $\pm$ 0.04
"Total Retention" (%) ^(a)	0.0011	0.0029	0.091	0.0079
Excreted in Urine (%)	0.014 $\pm$ 0.003 ^(c)	0.79 $\pm$ 0.27	0.059 $\pm$ 0.041	1.7 $\pm$ 0.3
Total Absorbed	0.0011	0.79	0.15	1.7

a. "Total Retention" was estimated by adding the liver content to 23 times the femur content.

b. "Total Absorbed" was estimated by adding "Total Retention" to the amount excreted in the urine.

c. Plutonium in the urine of this group was assumed to have leached from the feces during collection and was not included in the estimate of "Total Absorbed" (see text).

was increased by both citrate and DTPA, the effect of citrate being greater than that of DTPA. When both chelating agents were present in the gavage solution, retention was greater than for DTPA alone but less than when citrate was the only chelate.

Plutonium excretion in the urine varied widely between groups, which encourages one to believe that the leaching of plutonium from the feces could not have contributed significantly to the urine plutonium content, except in the group with the lowest apparent urinary excretion - the nitrate group - where urinary excretion of plutonium was 10-fold higher than total retention and was probably due almost entirely to contamination from the feces. The much higher urinary excretion in the other groups must have represented plutonium that was absorbed and re-excreted. Total absorption in these groups was estimated as the sum of total retention plus urinary excretion.

CONCLUSIONS

The values obtained in the present study for the gastrointestinal absorption of plutonium nitrate and plutonium citrate, while lower by a factor of approximately two than those reported by Weeks, et al.,⁽²⁾ nevertheless confirm the fact that plutonium citrate is much more readily absorbed than the nitrate. The effect of DTPA chelation is to increase plutonium absorption by another factor of five over that observed with the citrate. Although chelation is known to improve

metal ion absorption by plants and in the mammalian gut,⁽⁸⁾ the mechanism by which this occurs is presently unknown. Since virtually all of plutonium absorbed by the DTPA-treated rats reappeared in the urine, it seems probable that the form which crossed the gut was the plutonium-DTPA chelate and that this chelate remained intact in the blood until excreted in the urine. As a result, although the amount of plutonium absorbed was increased 700-fold by the presence of DTPA in the gut, the amount of plutonium retained was increased by only a factor of two or three. Citrate, while less effective in increasing absorption, was more effective in increasing the amount retained. The factor contributing to both aspects of this finding was undoubtedly the lesser stability of the plutonium-citrate complex. Once the citrate complex crossed the gut wall, it was metabolized to an extent which prevented its excretion and allowed its retention by liver and skeleton.

The effect of citrate and DTPA combined was an interesting one which might repay further study. The effects of the two agents were not simply additive. Absorption was double that with DTPA alone - an apparent synergistic effect - but retention was held down to about the same proportion of the absorbed dose as with DTPA alone.

The results of this study suggest that inhaled or ingested DTPA may, indeed, increase the absorption of plutonium from the gut but that the ultimate retention of this absorbed plutonium will be only two or three

times greater than if DTPA had not been employed. One must balance this risk against the potential benefit of treatment in each particular case.

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EFFECTS OF IRRADIATING SKIN

This project has three primary objectives: (1) determination of the response of the skin to ionizing radiation as influenced by size of exposure site and magnitude of radiation dose administered; (2) determination of the effect of age at time of exposure; and, (3) determination of the effects of combined treatment of the skin with radiation and with compound of known carcinogenic properties. The accompanying report describes progress toward objectives (1) and (2) in studies with miniature swine. Studies toward these objectives are also in progress in rats, and protocols have been established for the exposure of pigs to a combined treatment with radiation and carcinogens.

EFFECTS OF IRRADIATING SKIN**Investigator:***D. D. Mahlum***Technical Assistance:***Joan O. Hess*

Observation of pigs, which were exposed to 1-40 krad of beta radiation from ^{204}Tl plaques approximately 3 years ago, continues. Areas that were exposed to 6, 15, or 40 krad when the animal was an adult appear as thin whitened circles with an area only slightly larger than that originally exposed. Areas which received 1 or 2.5 krad are not grossly visible. In contrast, in animals exposed as neonates, lesions were noted at 2 krad and the affected area was several times that originally irradiated.

Additional analyses have been obtained from tissues from pigs which received ^{239}Pu intradermally 8-10 years ago. No skin tumors have yet been found. The concentration of ^{239}Pu in liver was as great or greater than that in bone. Estimated radiation dose to the liver ranged between 65 and 465 rads per year based on the concentration of ^{239}Pu in the liver at time of death.

The accessibility of skin increases the likelihood of its irradiation either through direct contamination of the surface or by penetration of skin with radioactive material. Ongoing studies in this laboratory have been examining both phases of the problem in the miniature pig. In addition, a small study is examining the response of the skin of the immature pig relative to that of the adult.

OBSERVATIONS

Approximately 3 years ago, young, adult, Hanford miniature pigs were irradiated with circular ^{204}Tl plaques with areas of 0.01, 0.5, 1.0, and 5.0 cm² (Annual Report, 1968). In addition, several areas on two pigs were

exposed to the ^{204}Tl plaques when the animals were 8 days of age. On those animals irradiated as young adults, sites exposed to the 0.5, 1.0, and 5 cm² sources at doses of 6.25, 15, and 40 krad are grossly visible as whitened areas. The size of the affected area is only slightly larger than that originally irradiated. In contrast, those animals exposed as neonates have grossly visible lesions at a dose of 2 krad and the lesions are several times the original size.

A study of the effects of ^{239}Pu administered to the pig intradermally was initiated approximately 10 years ago. As these animals came to necropsy, samples of tissues were obtained for analysis of radioactivity

as well as for histopathologic examination. Of major interest has been the relative concentrations of ^{239}Pu in bone and liver since these organs contain the major fraction of the residual ^{239}Pu with only a small fraction remaining in the skin. Values for liver and long bone for five animals that died between 1968 and 1971 are shown in Table 1. Although the amount of ^{239}Pu administered varied widely with one exception, the values for liver are higher than for bone. Radiation doses to the liver based on these concentrations range from 65 to 465 rads per year. These data obtained over a 3-4 year period suggest that a significant fraction of the intradermally injected ^{239}Pu is

translocated to liver and bone and is retained for relatively long periods.

CONCLUSIONS

As judged by appearance of gross lesions, the skin of immature swine reacts more vigorously to radiation insult than does that of the adult. It remains to be seen whether irradiation during the neonatal period is also more oncogenic than irradiation at later times.

The intradermal injection of ^{239}Pu into swine results in a greater concentration in the liver than in bone. Only a small fraction of the ^{239}Pu remained in skin.

TABLE 1. comparison of Level of ^{239}Pu in Liver, Bone, and Skin of Some Miniature Swine Injected Intradermally with ^{239}Pu

<u>Number</u>	<u>Date of Death</u>	<u>μCi Injected</u>	<u>dpm/g Tissue</u>		<u>% of Injected Pu</u>		
			<u>Liver</u>	<u>Bone</u>	<u>Skin</u>	<u>Liver</u>	<u>Bone</u>
1	1/7/68	25	4,800	470	0.08	9.11	2.91
2	3/5/68	100	10,650	6,450	0.93	5.60	11.71
3	3/12/68	25	3,350	980	0.82	9.00	4.36
4	2/5/71	45	1,580	1,850	0.55	3.76	(a)
5	11/17/71	25	2,680	1,250	0.17	9.44	(a)

a. Total bone was not analyzed

RADIATION EFFECTS ON CHYLOMICRON SYNTHESIS AND CAPILLARY PERMEABILITY
IN MICE

This project consists of two studies of radiation effects at the sub-cellular level employing techniques of autoradiography and electron microscopy. These effects are not only of basic interest in themselves, but it is anticipated that the systems studied may serve as models for testing the effects of radiation protective agents. Our studies of the effects of radiation on capillary structure and function are supplemented by parallel studies on the effects of chemical mediators, supported by the National Institutes of Health.

RADIATION EFFECTS ON CHYLOMICRON SYNTHESIS

Investigators:

J. C. Hampton and B. Rosario

C57 brown mice were exposed to 2500 R whole-body X-irradiation and sacrificed 1, 2, 3, 3.5, and 4 days post-irradiation. Thirty minutes prior to sacrifice, each mouse received by stomach tube 0.2 ml safflower oil containing 1.3 mc ^3H -palmitic acid. Specimens of ileum, jejunum, and liver were prepared for study in the electron microscope and for light microscopic autoradiography. The results showed that during days 1 and 2 postexposure, absorption and transport of lipid were essentially normal, and that the lipid and glycogen content of hepatocytes remained stable. Beginning on day 3, absorption from the ileum began to fail rapidly whereas the jejunum functioned at nearly normal levels. On day 3.5, jejunal absorption declined somewhat; and hepatocytes were virtually depleted of glycogen and of lipid droplets. Decreased absorption in the gut paralleled subcellular changes in the epithelium viz abnormal mitochondria and loss of ribosomes. The mice ate very little, if anything, after the beginning of day 3; and, usually, under conditions of starvation, the liver accumulates mobilized lipid; livers of these animals did not. These experiments did not reveal whether mobilization of stored fat was inhibited or whether the liver was unable to accumulate either mobilized lipid or chylomicrons.

Chylomicrons are the transport form of absorbed fatty acids and they are best characterized as spherical triglyceride protein complexes ca. 0.1-0.5 μm in diameter. Among the radiation effects reported in mice and rats were decreased absorption of fats, as observed in the electron microscope, but essentially normal plasma levels of fatty acids as observed in physiologic experiments. Epithelial cells, present on intestinal villi at 3 or more days after exposure to 1500 R X-rays, are severely deficient in ribosomes in the ileum and moderately to severely deficient in

the jejunum, suggesting that such cells would not be capable of synthesizing chylomicrons, at least in normal amounts. Ribosomes are essential to the synthesis of the protein moiety of chylomicrons and to the synthesis of proteins comprising the enzyme systems required in the uptake of fatty acids and monoglycerides from the gut lumen, and their subsequent reesterification to triglycerides. The extent to which liver damage contributes to continued normal levels of plasma lipids in irradiated animals has not been established. These experiments were an

attempt to reconcile some of the contradictions by following the uptake and transport of labeled fatty acids in ileum, jejunum, and liver autoradiographically and relating the findings to observations on the fine structure of these organs as revealed by electron microscopy.

OBSERVATIONS

Thirty-one, C57 brown mice, 12-15 g body weight, were used in two separate experiments. Five mice served as controls and 26 were exposed to 1500 R whole-body X-irradiation. Thirty minutes before sacrifice each mouse received 0.2 ml of safflower oil containing 1.3 mc ^3H -palmitic acid.

Absorption of the labeled palmitic acid-safflower oil mixture and chylomicron synthesis proceeded in normal fashion during the first 2 post-irradiation days; livers of these animals showed little change except perhaps a slight decrease in glycogen in hepatocytes and some accumulation of cell debris in reticuloendothelial cells lining sinusoids. Branches of the portal vein and most sinusoids were labeled in autoradiographs and most hepatocytes contained lipid vacuoles, some of which were labeled (Figures 1-7). Decreased absorption in the ileum was well-established by day 3, a time when villus shortening and depletion of the proliferative cell compartment were clearly evident. Some epithelial cells were labeled but large "clear" cells were present and un-

labeled. Electron microscopic images of such cells showed a sharp reduction in the number of ribosomes in the "clear" cells, indicating that metabolic pathways were no longer active. The numbers of these cells increased rapidly through 3.5 days, after which villus denudation began. At 3- to 3.5-day intervals, large lipid droplets were observed in some of the clear cells, but this was also seen at the villus tips in controls. It appears that degenerative cells cannot exclude from their cytoplasm large droplets of unhydrolyzed fats since the large intracellular droplets were similar in all respects to unhydrolyzed droplets in the gut lumen (Figures 8 and 9).

The events observed in the ileum also occurred in the jejunum but to a lesser degree. Even at day 4, villi were still present; some cells on villi did continue to function; chylomicrons were being synthesized and taken up by lymph vessels. Degenerative changes in cells were consistently found in the jejunum but did not necessarily become the dominant feature.

The livers showed no striking change in mice killed through the end of the second postirradiation day. Some reticuloendothelial cells contained cellular debris, and there was evidence of endothelial damage, but lipid was being picked up by the liver as evidenced by fat vacuoles in the cytoplasm and obvious label in cells and vascular lumens. By day 3, glycogen depletion was clearly evident and virtually complete in most cells in mid-lobular zones. On days 3.5 to

560X

FIGURE 1. Autoradiograph of a Cross Section of a Jejunal Villus 3.5 Days Postirradiation [Most epithelial cells are labeled as is the lacteal (L). Capillaries (C) are open and show no evidence of congestion.]

560X

FIGURE 2. Autoradiograph of an Ileal Villus from the Same Animal [Labeling is sparse over the epithelium, capillaries (C) are congested, and the lacteal (L) is open.]

910X

FIGURE 3. A Section of Liver Fixed 1 Day Postirradiation [Glycogen (G) is present in normal amounts and the cells appear normal.]

910X

FIGURE 4. A Section of Liver Fixed 4 Days Postirradiation (Glycogen has been depleted and the cells appear vacuolated.)

7,005X

FIGURE 5. An Electron Micrograph of Jejunum Fixed 1 Day Postirradiation [The epithelium appears normal; lipid absorption, transport, and chylomicron synthesis is normal as evidenced by lipid (L) droplets in cells and chylomicrons (C) between epithelial cells and in lamina propria.]

8,530X

FIGURE 6. Jejunum at 2 Days Post-irradiation [Chylomicron synthesis was still proceeding in normal fashion. Dense bodies seen here in the bases of epithelial cells (D) are indicative of impending degenerative cell change.]

12,200X

FIGURE 7. The Lamina Propria of a Jejunal Villus at 1 Day Postirradiation [Contains large numbers of chylomicrons, most of which are found in a lymph vessel (lacteal) in this picture (L). Two blood capillaries (C) are shown and it is clear that their endothelial linings form an effective barrier to chylomicrons.]

12,200X

FIGURE 8. Ileal Epithelial Cells Fixed 3 Days Postirradiation [In the ileum at this time and to some extent in the jejunum 0.5-1 day later, epithelial cells continue to take up lipid (L) but do not make chylomicrons nor discharge the lipid into intercellular spaces. The lipid is usually in the form of large droplets, often larger than the cell nucleus. Although a few free ribosomes (R) can be seen in the cytoplasm, endoplasmic membranes are essentially devoid of them.]

12,200X

FIGURE 9. An Ileal Cell Which, in Addition to Depletion of Ribosomes, is Free of Lipid [Mitochondrial inclusions (M) and short, stubby microvilli (MV) at the cell surface, also shown in Figure 8, are characteristic of villus cells at this postirradiation interval.]

4, both lipid and glycogen were essentially depleted; and mitochondria, although appearing normal in structure in electron micrographs, had a vacuolated appearance when observed under the light microscope (Figures 10 and 11).

CONCLUSIONS

The results of this work, thus far, show a close correlation between the fine structure of intestinal epithelial cells and their ability to carry out certain metabolic reactions required for the transport into epithelial cells of digested lipids, for reesterification of absorbed fatty acids, and for the ultimate synthesis and transport of chylomicrons. Cells that were deficient in ribosomes showed failure in these metabolic pathways. Previous work by others has shown that puromycin inhibits the synthesis of chylomicrons by epithelial cells through interference with protein synthesis. Experiments now underway in this laboratory using ^3H -leucine as a label rather than ^3H -palmitic acid will show whether radiation induces a similar effect on protein synthesis. The work described above suggests that cells which were essentially mature at the time of irradiation could still make chylomicrons and, therefore, the necessary protein. Cells not yet mature, but postmitotic, seem to be unable to support such metabolic functions for very long periods of time probably because they are deficient in the quantity and/or quality of ribosomes.

Mice exposed to doses of X-rays used in these experiments eat little

6,710X

FIGURE 10. Liver Fixed 2 Days Post-irradiation [Liver cells appear normal and contain glycogen (G) and lipid (L) at approximately normal levels. Sinusoidal lumens (S) contained variable quantities of cell fragments and occasional lipid droplets (LD) were found in the perivascular spaces.]

or nothing, beginning sometime between 48 and 72 hr postirradiation, producing, in effect, a starved animal. This accounts for the observed glycogen depletion in liver but produces a paradox with respect to lipid accumulation in hepatocytes, since starved animals show an increase in liver lipid resulting from accumulation of mobilized body fat. Livers of these mice showed a sharp decline in liver lipid, beginning on day 3. This finding cannot be adequately explained on the basis of these experiments. Chylomicrons were present in good

8,530X

FIGURE 11. Liver Fixed 4 Days Post-irradiation [Sinusoids (S) are free of congestion from either cell debris or blood cells and the endothelial linings are intact but macrophages (RE) are hypertrophied. The hepatocytes are devoid of glycogen and, as seen here, the vacuolated appearance shown in Figure 4 is attributed to mitochondria, somewhat larger than normal, but also unmasked by glycogen depletion. There is some lipid (L) in the cells but it is extremely dense and irregular in profile, more characteristic of endogenous lipid containing granules associated with degenerative processes rather than that of active metabolic pools shown in Figure 10.1

numbers in jejunal lymphatics but apparently were not removed from plasma by the liver. This, I believe, helps to explain why plasma levels of absorbed lipid remain near normal after irradiation.

RADIATION EFFECTS ON CAPILLARY PERMEABILITY IN MICE

Investigators:

J. C. Hampton and B. Rosario

C-57 brown mice were exposed to 1500 R whole-body X-irradiation and sacrificed at 1, 2, 3, and 3.5 days postexposure. Each animal was injected intravenously with horseradish peroxidase (HRP) 1 to 2 min before sacrifice and specimens of brain, lung, heart, skeletal muscle, adrenal gland, pancreas, intestine, and kidney were prepared for light and electron microscopic localization of HRP. Unfenestrated capillaries showed two types of damage: (1) uniform blackening of the entire thickness of endothelial cell cytoplasm with HRP reaction product and (2) failure of active transport as judged by the lack of evidence of pinocytotic activity. Similarly, two changes in fenestrated capillaries were observed: (1) discontinuities in the endothelial lining which appeared to be the result of rupture and (2) blackening of endothelial cytoplasm by HRP reaction product. Because of the extreme thinness and fragility of the fenestrated vessels, interpretation at this stage is only a guarded opinion. Extreme staining of the internal elastic lamina by HRP reaction product in kidney arterioles remains unexplained. Experiments on mesenteric arteries and aortae in irradiated animals were carried out; and similar but less intense reactions could be found in mesenteric arterioles, but not in the main trunks of mesenteric arteries nor in aortae.

This study is based upon established differences in capillary fine structure which permit classification of capillaries according to the nature of the endothelial lining, the presence or absence of a basement membrane, and the presence or absence of a complete investment of the capillary by a closely adhering layer of cells which comprise the organ in which the capillary is located. For purposes of this study, two types of endothelium are being considered: (1) unfenestrated endothelial cells - those which are fairly thick and in which transepithelial transport is believed to occur by vesicle formation

at the cell surface (pinocytosis) followed by transfer of the vesicle to the opposite side of the cell and discharge into extravascular spaces; (2) fenestrated endothelial cells - those in which cytoplasm is extremely thin and perforated at regular intervals by pores 60 nm in diameter and spanned by a delicate layer of mucopolysaccharide. The pores constitute the main avenue of transfer across the cell. Attachments between endothelial cells are of two types, the gap junction characterized by a 2 nm space between apposing cell membranes and the tight junction in which the outer lamellae of adjacent unit membranes

are fused. Some organs, e.g., heart, skin, and skeletal muscle, contain only unfenestrated capillaries; others, such as intestine, contain both fenestrated and unfenestrated capillaries but in very restricted locations; and still others, e.g., kidney, contain only fenestrated capillaries. All of these capillaries are surrounded completely by a basement membrane. Fenestrated capillaries are normally more permeable than nonfenestrated capillaries. The third type of capillary being studied occurs only in brain. It consists of an unfenestrated endothelium, a basement membrane, and closely applied processes of glial cells, a supporting but not a true nerve cell in the central nervous system. This type of capillary is deemed to be the least permeable, at least the most selectively permeable, capillary type in the mammalian vascular system.

The use of suitable tracers provides a way by which exchange between blood vessel lumens (plasma) and extravascular spaces can be studied with light and electron optics. Horseradish peroxidase (HRP), after intravenous injection, can be localized through histochemical reactions; and because its size approximates that of plasma proteins, it can be used as a sensitive indicator of changes in capillary permeability induced by irradiation and by chemical mediators. Variations in capillary fine structure occur normally in mammals, depending upon the organ studied, and vascular permeability varies according to the type of vessel and size of the tracer. Permeability changes have been reported to occur in

some types of capillaries and larger vessels in a few organs and tissues and under a wide variety of experimental conditions, but no attempt has been made to study the response of several given types of vessels in a selected group of organs under controlled conditions. Thus, it is not known to what extent observed changes in permeability should be attributed to a particular type of vessel, to the experimental conditions imposed, or to the organ under study being particularly vulnerable to damage. These experiments are intended to determine the effects of X-irradiation on the microvasculature in heart, lungs, brain, skeletal muscle, adrenal, pancreas, small intestine, and kidney. The results will be compared with effects of chemical mediators (bradykinin, histamine, and compound 48/80, a histamine releaser) on the vessels of the same organs in a study supported by NIH.

OBSERVATIONS

Thirty-three C57 brown mice were exposed to 1500 R whole-body X-irradiation and sacrificed at 1, 2, 3, and 3.5 days postexposure. Unirradiated mice were used as controls. Each mouse was injected with HRP 1 to 2 min before sacrifice and tissue slices were incubated with 3-3' diaminobenzidine containing a small amount of H_2O_2 as a substrate to develop the black reaction product used to localize HRP.

Although this work has been underway for nearly 2 years, meaningful data on capillaries are only now becoming available. Of the organs examined, it appears that except for the kidney no single

type of capillary is more vulnerable than another. Both fenestrated and unfenestrated capillaries show increased permeability. Discontinuities in the endothelium appeared more frequently in fenestrated capillaries particularly in the kidney adrenal gland, and pancreas; but the endothelial linings of these vessels are so thin and probably extremely fragile that part of the damage could be attributed to handling during the fairly complicated histochemical procedures. Changes in permeability fall into two major categories: (1) rupture of the vessel, which was limited to fenestrated capillaries and (2) failure of the endothelial cells to exclude HRP from their cytoplasm so that there appeared to be little control of leakage across the cell. Postcapillary venules, known to be more permeable to carbon black and other particulates, seemed to be no different in their response to irradiation than unfenestrated capillaries. Arterioles showed intense localization of HRP in the internal elastic lamina, a finding which in the extreme degree is limited to kidney and gut. In order to study this aspect of the problem, mesenteric arteries and aortae were examined in rats and mice after exposure to 4 kR ^{60}Co gamma rays and to 1500 R X rays. Similar but much milder reactions were observed in mesenteric arterioles but not in the elastic lamina of the main trunks of the mesenteric arteries nor in the elastic laminae of the aortae.

CONCLUSIONS

HRP crosses unfenestrated capillaries mainly in vesicles formed by endocytosis (pinocytosis), by transfer across the cell and discharge into perivascular spaces by exocytosis, and, to a lesser extent, by traversing gap junctions between endothelial cells. Evidence of damage to this type of vessel was either uniform blackening of the cytoplasm of the cell or lack of any evidence of HRP in vesicles. The latter probably represents an early manifestation of damage and, the former, a somewhat later one. Assessment of damage in unfenestrated capillaries is much easier because a transport pathway involving the membrane is known and because the unfenestrated endothelial cell is 5 to 10 times thicker than fenestrated cells. Damage to the delicate fenestrated endothelial cells is much more subjective since one must often base a decision upon apparent electron density, which can vary significantly by a slight increase in section thickness, and upon discontinuity or rupture of the lining cells themselves and/or loss of the attenuated diaphragms which normally cover the fenestra. In either case, observations must be carefully compared to images of tissues prepared in the more conventional manner for electron microscopy to avoid misinterpretation of artifacts which might arise as a result of the histochemical techniques required to demonstrate HRP in tissues. This is a

time-consuming but necessary exercise since there seems to be no other way to deal with the problem of the fenestrated capillary.

Capillaries in brain are, with the exception of the area *postrema* and one or two other areas deep in the brain, of the unfenestrated type; and these vessels are tightly enclosed by glial cells separated from the endothelium by a basement membrane. Some darkening of endothelial cytoplasm in brain has been observed but no extravasation of HRP into sur-

rounding brain tissue was observed to occur; so, it is reasonable to postulate that at least with respect to HRP the blood-brain barrier was functionally intact. The kidney contains mostly fenestrated capillaries, and this was the only organ in which the endothelial cells seemed to be uniformly damaged by the end of day 2 postexposure. This is also the organ in which arterioles most often showed intense staining of the internal elastic lamina. Why this should be true remains to be explained.

11,400X

FIGURE 1. Unfenestrated Capillary in Left Ventricle Fixed 1 Day after Irradiation [There is no evidence of accumulation of HRP, and the endothelium appears normal except for a paucity of pinocytotic vesicles (V) and an open intercellular junction (J).]

4,800X

FIGURE 2. Unfenestrated Capillary in Left Ventricle Fixed 2 Days Postirradiation [Most capillaries in heart observed at 2 days postirradiation and later showed numerous endothelial cytoplasmic projections and irregular large vacuoles (V). Perivascular spaces were frequently much wider than in controls.]

10,500X

FIGURE 3. Unfenestrated Capillary in Ventricle Fixed 2 Days Postirradiation [This capillary is lined by two cells; the junctions between them (J) clearly delineate each cell. The cytoplasm of each cell shows numerous pinocytotic vesicles (V); the dark cell also contains large vacuoles (V¹). Neither cell appears normal since the cytoplasm of the dark one is permeated with HRP reaction product, suggesting that it is completely permeable to HRP, and the light cell appears swollen and to have lost much of the cytoplasmic detail normally found in endothelial cells (compare with Figure 1). Small pinocytotic vesicles (V) in both cells seem to be devoid of HRP reaction product.]

1,700X

FIGURE 4. Unfenestrated Capillary in Adrenal Medulla Fixed 3 Days Postirradiation [The endothelial cells are somewhat darker than normal and usually show long protoplasmic extensions (P) projecting into the vessel lumen. The capillary is surrounded by chromaffin cells.]

6,875X

FIGURE 5. Fenestrated Capillary in the Adrenal Cortex Fixed 3.5 Days Postirradiation (The endothelial lining is intact; but, as shown here, cell debris was usually found in the lumens of cortical vessels.)

1,760X

FIGURE 6. Kidney Glomerulus Fixed 2 Days Postirradiation [Virtually all capillaries in kidney are fenestrated and frequently showed disruption of the endothelium. A portion of a glomerulus occupies the left one-half of this picture, and at X a peculiar reticular arrangement of endothelial cell processes can be seen. The glomerular epithelium (E) is uniformly more dense than the endothelium (EN). Capillary lumens (L) are filled with plasma and HRP, but urine (U) spaces appear relatively clear. A part of a glomerular artery (A) can be seen at the upper right, and intense staining of the internal elastic lamina (EL) is evident.]

6,875X

FIGURE 7. A Fenestrated Capillary in Kidney Fixed 3 Days Postirradiation [The vessel is bounded by a distal convoluted tubule on the left and a proximal convoluted tubule on the right and is separated from the tubules by a basement membrane (B). The endothelium is discontinuous (X), a common finding in kidney, adrenal gland, and pancreas after irradiation.]

9,900X

FIGURE 8. A Part of a Distal Convoluted Tubule in a Kidney Fixed 1 Day Postirradiation (Occasionally in control animals and with increasing frequency with time after irradiation, permeation of parenchymal cells by HRP was observed, particularly in kidney. Adjacent tubule cells interdigitate with each other at their lateral boundaries; this figure shows interdigitation of cell processes permeated with HRP with an apparently undamaged cell.)

4,785X

FIGURE 9. Kidney Fixed 3 Days Postirradiation [Blackened spaces between processes of tubule cells are shown at the right and at the lower left of the figure; a capillary filled with erythrocytes (RBC) is in the center. At the upper left is a section through the thin segment of the loop of Henle showing a blackened epithelial cell, processes of which interdigitate with a normal cell. Uniform staining of damaged cells has been reported to occur in most villus epithelial cells in the ileum at 3 days postirradiation; Figures 8 and 9 clearly demonstrate that permeability changes occur in parenchymal cells of other organs as well.]

6,600X

FIGURE 10. An Unfenestrated Capillary in Cerebral Cortex Fixed 3 Days Postirradiation (Although the endothelial lining of the capillary is partly blackened by reaction product, there has been no evidence to indicate escape of HRP, in appreciable quantities at least, into extravascular spaces in brain.)

3,575X

FIGURE 11. An Arteriole in Kidney Fixed 1 Day Postirradiation [The internal elastic lamina (EL) is densely stained with reaction product, as are some of the elastic fibers (EF) at the periphery of the vessel. No such reaction has been observed in unirradiated mice.]

2,860X

FIGURE 12. An Arteriole in Mesentery Fixed 3 Days Postirradiation [The internal elastic lamina (EL) in this vessel is more heavily stained with HRP than are unirradiated arterioles but much less stained than those observed in kidney and occasionally in the gut submucosa.]

3,025X

FIGURE 13. Aorta Fixed 3 Days Postirradiation [Only modest staining of the internal elastic lamina (EL) was found in irradiated mice even at day 4 when the animals were moribund.]

MINIATURE SWINE AS MODEL FOR INTRACAVITARY IRRADIATION

This is a new project, supported by discretionary funds during the latter months of fiscal year 1971. Medical interest in the use of ^{252}Cf as a neutron source in radiotherapy made it of critical importance to acquire data on the effects of such use under conditions simulating the intended applications in humans. The miniature swine appeared to be an excellent experimental subject for conducting such studies.

THE HANFORD MINIATURE SWINE AS AN ANIMAL MODEL
FOR INTRACAVITARY IRRADIATION BY RADIUM-226 AND CALIFORNIUM-252

Investigators:

M. F. Sullivan and J. L. Beamer

Techniques have been developed using the Hanford Miniature Swine that will allow a comparison of the effects of ^{226}Ra capsules implanted in the uterine cervix with those resulting from implanted ^{252}Cf afterloading capsules. These methods should allow calculation of an RBE for this neutron emitter.

The availability of the fast neutron-emitting isotope, ^{252}Cf , provides a radiation source with a flexibility that has heretofore not been available to those without access to neutron beams produced by reactors or generators. The compact size of the californium sources and their relatively low cost has opened a new application for the use of fission neutrons in radiotherapy by intracavitary and intrastitial implantation. The high LET (linear energy transfer) and low OER (oxygen enhancement ratio) predicates their superiority to conventional ^{226}Ra or ^{137}Cs encapsulated sources now being used for intracavitary treatment of cervical or uterine cancer where these radiation characteristics are advantageous.

Neutrons also present a potential risk, however, to organized epithelia of normal tissue, such as in the mucosa of the rectum or urinary bladder that may be irradiated during intracervical exposure. Since the dosimetry and effects of therapy by

radium sources are well known, the comparative effects (RBE, radiobiological effectiveness) under conditions that are analogous to those used clinically would be particularly useful for arriving at a safe but therapeutic dose for tumor therapy.

OBSERVATIONS

Although the anatomic relationships of the swine urinary bladder, cervix, and rectum differ from those in the human to some extent, the distance of these organs from a site in the uterine cervix where radiation sources can be placed is very similar. To place the ^{226}Ra and ^{252}Cf sources in the desired location, special polyethylene tandems 30 cm in length were used.* Around the anterior end of the tandem was placed an inflatable cuff that was filled with Hypaque**

* Radium Chemical Co., New York, N.Y.

** Winthrop Laboratories, Div. of Sterling Drug, Inc., New York, N.Y. 10016.

to locate its position radiographically and to hold it in the desired position. Afterloading techniques were used to place either the two 20 mg ^{226}Ra or four 12 μg ^{252}Cf

sources. Radiograms, both lateral and ventral, were made to confirm the source location and as an aid in calculating dose to various organs. An example is shown in Figure 1.

FIGURE 1. Radiogram of a Miniature Swine After a Barium Enema Showing a Tandem Implant Containing Four 12 μg ^{252}Cf Sources in the Uterine Cervix (An inflated rubber cuff filled with Hypaque secures the tandem in place. The urinary bladder is drained during exposure by a Foley catheter.)

The radiation doses administered were fractionated into two treatments divided by a 10-day recovery period. The initial radium exposures were for either 100 or 200 hr which were compared to 64 and 128 hr of ^{252}Cf implantation. The calculated doses at a reference point 2 cm lateral to the sources were 62 rads/hr for ^{226}Ra and 19.8 rads/hr for ^{252}Cf . The pigs were killed at either 7 or 14 days after the second exposures to determine the acute effects.

Gross observations of radiation damage from a 200-hr exposure to 40 mg of radium (approximately 12,000 rad) indicated that the rectum and the trigone of the bladder received severe injury. Photomicrographs of these tissues are shown in Figure 2. The mucosa of the anterior wall of the rectum was ulcerated but damage was localized to an area not much larger than that occupied by the sources in the adjacent cervical canal. The bladder mucosa showed hyperemia and edema of the trigonal mucosa with petechial hemorrhages. Under the light microscope, these changes showed the rectal mucosa to have received damage in the area of the ulcer equivalent to at least 2000 rad; but in this acute phase, the ulcer did not extend below the lamina propria. The bladder mucosa showed ballooning of epithelial cytoplasm.

Gross damage after exposures to about 2500 rad from ^{252}Cf appeared to be much less severe than after the first radium exposures. Although lower doses (6200 rad) of gamma radiation from radium were also administered, histopathologic data are not yet available to compare with neutron injury. The results obtained from the high initial exposures indicate the acute damage is similar in location to what might be expected in human radiotherapy. These studies are too preliminary, however, to attempt any evaluation of a dose response to either radium or californium. They do suggest that the swine is a useful animal model for evaluating the effects of an intracervical treatment with ^{252}Cf .

CONCLUSIONS

The organs evidencing most severe damage from intracervical placement of either ^{226}Ra or ^{252}Cf afterloading capsules were the rectum, urinary bladder, and the uterine cervix. Distance and anatomic relationship, as well as the response of these organs to exposure doses within the range used for treatment of cervical cancer, indicate that the miniature swine is a useful animal for evaluating the potential usefulness of intracavitary radiation therapy sources.

FIGURE 2A. The Rectal Mucosa 7 Days after a 200-Hr Exposure to 40 mg of ^{226}Ra Showing a Radiation Ulcer Bordered by Mucosa That Showed Much Less Damage (Note the perivascular changes in the submucosal connective tissue.)

FIGURE 2B. Epithelium of the Trigone of the Urinary Bladder 7 Days after a 200-Hr Exposure to 40 mg of ^{226}Ra (The cells of the transitional epithelium show large intracytoplasmic vacuoles but no ulceration was seen.)

CELLULAR REGULATORY MECHANISMS

The cellular and molecular level studies included in this project are directed toward the elucidation of mechanisms involved in regulation of enzyme synthesis and growth. Present studies are concentrated on the cellular control mechanisms in a fairly simple but representative eukaryotic cell, Neurospora crassa. While previous concepts developed from bacterial studies are useful in the design of experimentation with higher cells, the simplistic bacterial models are not adequate to explain the regulation that occurs in higher organisms by such mechanisms as product inhibition, control of nutrient entry, synthesis and rate of synthesis of enzymes, degradation of enzymes, covalent modification of enzymes, etc.

These studies also receive substantial support from the Battelle Institute. No effort is made to separately identify AEC- and Battelle-funded studies. Combining their support permits a broader program of research than could be realized by separate efforts on behalf of the two sponsors.

INDUCTION OF A PLASMA MEMBRANE-BOUND Co^{2+}
ACTIVATED AMINOPEPTIDASE IN NEUROSPORA

Investigators:

*W. R. Wiley, R. B. Sanders,
and Laura S. Winn*

A membrane-bound Co^{2+} activated aminopeptidase activity has been identified in Neurospora crassa. The enzyme(s) is induced when cells are grown in the presence of a medium containing 1% peptone. Induction of the enzyme(s) is repressed by sucrose. The membrane-bound enzyme(s) differs from the intracellular aminopeptidases in that the intracellular enzymes are constitutive and do not increase significantly during growth in peptide-containing medium. The enzyme has a broad specificity hydrolyzing a number of di- and tripeptides from the N-terminal end of the peptides.

Previous studies on the transport of amino acids by cells of Neurospora crassa showed that di- and tripeptides were cleaved to single amino acids before transport across the cell membrane in Neurospora. The implication of these results was that aminopeptidases associated with the plasma membrane or localized in the periplasmic spaces of the cell were involved in the cleavage of the peptides. In the previous Annual Report, it was shown that low levels of a Co^{+2} -requiring aminopeptidase was intimately associated with purified plasma membrane vesicles prepared from spheroplasts of Neurospora. In this report, data are provided to show that when cells are grown in a medium containing peptides as the sole carbon source, the membrane-bound aminopeptidase was induced while the intra-

cellular (soluble) aminopeptidase level remained constant. The membrane-bound aminopeptidase is repressed by growth in medium-containing sucrose. The enzyme(s) has a fairly broad specificity, releasing a number of neutral amino acids from the N-terminal end of di- and tripeptides.

OBSERVATIONS

Table 1 shows a comparison of the levels of cell membrane-bound and soluble aminopeptidase activities in cells grown on various media. The synthetic substrate, leucine-p-nitroanilide (LNA), was used to assay for the aminopeptidase activity in these experiments. Casitone and peptone are commercial designations for media high in low molecular weight peptides. As shown in Table 1, growth

of the cells in media containing peptides resulted in a 4- to 5-fold increase in membrane-bound leucine aminopeptidase while the soluble aminopeptidase activity remained relatively constant. The presence of sucrose in the medium appears to inhibit this increase in aminopeptidase activity. The increase in membrane-bound aminopeptidase activity is correlated with the presence of peptides in the medium since free amino acids (acid hydrolyzed casein) did not increase the level of the membrane-bound activity (Table 1).

To differentiate between bound and soluble aminopeptidase activity, spheroplasts were prepared from induced cells by removal of the cell wall. The spheroplasts were washed three times in 0.80M NaCl buffer and lysed by mild osmotic shock. The soluble and particulate fractions were separated and washed five times in

0.15M NaCl by differential centrifugation (3000 *g* - 37,000 *g*). Washing in NaCl removes some membrane proteins which are soluble in 0.15M NaCl. All supernatant fractions (including washes of the membranes) were combined and constituted the soluble aminopeptidase activity fraction. Pellet fractions sedimenting at 37,000 *g* constituted the membrane fraction. Microsomal and mitochondrial fractions and nuclei were removed from the cell membrane fractions by discontinuous sucrose gradient centrifugation.

The cell membrane-bound aminopeptidase activity is associated with closed membrane vesicles. As shown in the electron micrograph, Figure 1, the closed membrane sacs are surrounded by a single trilaminar membrane layer which is 70-90 Å thick. The vesicles appear empty and devoid of internal structure.

TABLE 1. Comparison of Levels of Membrane-Bound and Soluble LNAase Activity in Cells Grown on Various Media ^(a)

Medium	Doubling Time, hr	^(b) Units/mg Dry Weight Cells	
		Membrane-bound	Soluble
Mineral salts + 2.0% sucrose	3.0	68	673
Mineral salts + casitone	4.0	350	700
Mineral salts + peptone	4.0	400	733
Mineral salts + peptone + 2.0% sucrose	3.5	99	713
Mineral salts + acid hydrolyzed casein	3.0	70	733

a. Cells germinated and grown for 9 hr on the respective media

b. nmoles LNA cleaved/hr/mg dry weight cells

51,200X

FIGURE 1. Electron Micrograph of Cell Membrane Vesicles Prepared from Spheroplast of Neurospora crassa

The data shown in Table 1 suggest that the cell membrane-bound aminopeptidase activity may be inducible while the soluble form of the enzyme is constitutive. To test this possibility, we examined the time course of synthesis of the two types of enzymes in logarithmically growing cells of Neurospora.

Figure 2A shows the time course of formation of the particulate enzyme(s) following transfer of the cells from mineral salts sucrose medium to peptide-containing medium. At zero time (arrow), washed, germinated conidia were added to fresh mineral

salts medium containing peptides with and without sucrose. In the presence of sucrose, the units of aminopeptidase activity remained fairly constant for 8 hr. In the absence of sucrose, however, there was an increase in the rate of formation of membrane-bound activity. At zero time, the membrane-bound activity constituted 10% of the total activity of the cell. After 8 hr, the membrane-bound activity constituted approximately 32% of the total aminopeptidase activity of the cell. In other experiments, this value was as high as 55%.

FIGURE 2A. Time Course of Membrane-Bound Aminopeptidase Biosynthesis in Peptide-Mineral Salts Medium (O-----O without sucrose; ●-----● with 2% sucrose.)

FIGURE 2B. Time Course of Soluble (intracellular) Aminopeptidase Synthesis in Peptide-Mineral Salts Medium (O-----O without sucrose; ●-----● with sucrose.)

Figure 2B shows that synthesis of the soluble enzyme is not altered by the presence or absence of sucrose in the peptide-mineral salts medium. These results are consistent with the interpretation that the membrane-bound aminopeptidase activity is inducible by peptides in the external medium and that the induction of this activity is repressed by sucrose.

The cell membrane-bound enzyme(s) hydrolyzes a number of di- and tripeptides in addition to the synthetic substrate LNA. A list of di- and tripeptides which are hydrolyzed by the

particulate enzyme(s) is given in Table 2. It is apparent that the enzyme, if indeed it is a single enzyme, has a broad range of specificity, hydrolyzing a number of neutral amino acids from the N-terminal end of the peptide. While preliminary data failed to show differences in specificity between the soluble and particulate enzyme, additional work will be necessary to substantiate this observation.

Most aminopeptidases are metallo-enzymes, i.e., requiring a metal enzyme complex for activity. The

TABLE 2. Rates^(a) of Hydrolysis of Various Peptides by a Membrane-Bound Aminopeptidase from Neurospora

Peptide	Concentration (mM)	Rate ^(b)
Glycylglycine	8.5	85
L-Leucyltyrosine	8.5	115
L-Leucyl-leucine	8.5	115
L-Leucyl-p-nitroanamide	1.7	15.25
L-Leucyltyrosine + 1×10^{-3} M EDTA	8.5	2

a. Rate of hydrolysis measured titrimetrically using a radiometer titrator

b. Rate = nmoles acid equivalents released/min

membrane-bound aminopeptidase described in this report appears to be a Co^{2+} metallo-enzyme. When the membrane preparations are dialyzed against ethylenediaminetetraacetic acid (EDTA), 10^{-3} M, the enzyme loses approximately 90% of its activity. The inhibition can be reversed by the addition of 7.5×10^{-4} M CoCl_2 to the reaction mixture. Cobalt ions can be replaced completely or partially by other metals ($\text{Co}^{2+} > \text{Mn} > \text{Mg}$). Published observations suggest that metal replacement in aminopeptidases may alter the specificity of the enzyme. We are presently investigating this possibility for the Co^{2+} -requiring aminopeptidase from Neurospora.

CONCLUSIONS

The function of aminopeptidases in cells has not been fully established; however, three roles for these peptidases in cells have been proposed: (1) they act to protect the cells from the deleterious effects of uncleaved peptides arising from in vivo biosynthesis of peptides, (2) they function in the maturation of newly synthesized proteins by limited digestion of the N-terminal peptides of the proteins, and (3) they serve a nutritional role by digestion of exogenous aminopeptidases for transport and metabolism inside the cell.

The third proposal is consistent with the finding of an inducible membrane-bound aminopeptidase whose control is linked to sugar metabolism. Since the function of the enzyme is to cleave peptides at the external surface of the cell, it seems reasonable to conclude that it is localized on or near the external surface of the membrane. If future experiments can substantiate this conclusion, it should be possible to use this enzyme to: (a) investigate the synthesis and incorporation of a single protein into the external surface of the cell membrane and (b) differentiate between the outer and inner surfaces of the cell membrane. Studies along these lines are presently underway.

PHYSIOLOGICAL STUDIES OF TRP-2e MUTANT IN NEUROSPORA CRASSA

Investigator:

J. R. Turner

Technical Assistance:

Lucille M. Butcher

The trp-2e mutation does not impose any nutritional requirement on N. crassa but results in excretion of phosphoribosyl anthranilic acid (PRA). PRA was identified by cross-feeding to tryptophan auxotrophs. Induction of kynureninase synthesis by tryptophan is severely limited in this mutant although transport and pool formation are not affected. The specific activity of the biosynthetic enzymes are equal to levels found in wild type. PRA excretion appears to result from a change in the feedback regulation of the biosynthetic pathway.

We reported (Annual Report, 1970) isolation of a unique mutant in Neurospora trp-2e, which is prototrophic and excretes anthranilic acid. A change in the regulation of either biosynthesis or degradation of tryptophan, as a result of mutation, could explain this phenotype. We have identified a second excretion product which allows a more definitive description of the effects of this mutation.

OBSERVATIONS

Strains of Neurospora which contain the trp-2e mutation grow well in unsupplemented salts medium. Growth rates of the mutant are not different from wild type; however, during growth, fluorescent material appears in the growth medium of the mutant. Figure 1 (Panel A) shows a typical

growth curve for the trp-2e mutant in minimal medium. Panel B shows the appearance of ethyl acetate-extractable fluorescent material during the same time period. The fluorescent material accumulates during the log phase of growth and subsequently disappears. Paper and thin layer chromatography of aliquots of the growth medium or ethyl acetate-extractable material showed anthranilic acid to be the first fluorescent product detectable as a function of time. Subsequently, a series of compounds appear in the filtrate, none of which could be identified chromatographically with products expected to arise from the degradation of tryptophan (kynurenine, formyl-kynurenine, formyl-anthranilic acid, hydroxy-kynurenine, N-acetyl-kynurenine, or nicotinic acid). Further, assay of

FIGURE 1. Panel A. Increase in Dry Weight per 100 ml of a Culture of Strain 92814-1 (trp-2e) Incubated in Minimal Medium. Panel B. Ethyl Acetate Extractable Anthranilic Acid in Culture Filtrate of Strain 92814-1 (trp-2e).

crude extracts of these cells confirmed that tryptophan pools were comparable to wild type cells grown on minimal medium. Thus, these products are derived from the biosynthetic pathway and are not a result of overproduction of tryptophan followed by degradation via the normal degradation pathway.

The growth medium was examined for biosynthetic intermediates by classical cross-feeding experiments wherein specific tryptophan auxotrophs were inoculated into solid minimal medium which was then spot-inoculated with trp-2e culture. The close proximity of the excreter mutant and aux-

otrophs allows detection of compounds which normally would be chemically degraded by the acid pH (5.8) of the medium. Using this cross-feeding method (Table 1), the primary excretion product was shown to be phosphoribosyl anthranilic acid (PRA), the second intermediate in the biosynthetic pathway, since it supports growth of a trp-2 (AAase) or trp-4 (PRTase) mutant but not a trp-1 (PRAiso) or trp-3 (TRPase) mutant. The chromatographic identification of anthranilic acid but not PRA probably was due to the rapid conversion of PRA to anthranilic acid known to occur at pH 5.8.

TABLE 1. Cross-Feeding Experiment

Mutation:	trp-1	trp-2	trp-3	trp-4
Enzymatic defect:	PRA isomerase IGP synthetase	AAase	TRPase	PRTase
Product required for growth	IGP tryptophan	Anthranilate PRA CDRP IGP tryptophan	tryptophan	PRA CDRP IGP tryptophan
Growth on trp-2e filtrate:	-	+	-	+

Legend Table 1

AA; anthranilic acid

PRA; N-(5'-phosphoribosyl)-anthranilate

CDRP; 1-(0-carboxyphenylamino)-1-deoxyribulose-5-phosphate

IGP; indole-3-glycerol phosphate

Overproduction of biosynthetic intermediates has been shown to result from either excess or decreased synthesis of enzymes or change in feedback sensitivity of regulatory enzymes. Crude extracts of trp-2e cells grown in minimal medium were assayed for anthranilate synthetase (AAase), anthranilate-5-phosphoribosylpyrophosphate phosphoribosyl transferase (PRTase), indole glycerolphosphate synthetase (IGPase) and tryptophan synthetase, four of the biosynthetic enzymes. All enzymes were found (Table 2) at specific activities equal to those in wild type cells.

The ability of this strain to degrade tryptophan was examined by as-

saying induction of kynureninase when tryptophan was supplied in the growth medium (Figure 2). Despite the presence of intracellular levels of tryptophan in trp-2e greater than that of wild type (Panel A), the rate of induction of kynureninase synthesis (Panel B) is at a much slower rate than wild type. Wild type rate is approximately two times greater than the maximum trp-2e rate. However, examination of the trp-2e growth medium showed formyl-anthranilic acid present which indicates that tryptophan degradation is proceeding in a normal fashion, that is, tryptophan+formyl-kynurenine $\rightarrow$ formyl-anthranilate.

TABLE 2. Specific Activity of Biosynthetic Enzymes in Wild Type and trp-2e

Enzyme	Specific Activity (units/mg dry weight)	
	trp-2e	Wild type
AAase	0.0138	0.0158
PRTase	0.0117	0.0127
IGPase	0.0044	0.0061
TRPase	0.0692	0.0625

CONCLUSIONS

Excretion of PRA by trp-2e strains is not due to excess synthesis of any of the four biosynthetic enzymes assayed. Conversely, reduced synthesis of the one enzyme not assayed (PRA isomerase) seems unlikely since the mutant exhibits a growth rate identical to wild type. Degradation of tryptophan appears normal although kynureninase induction is reduced from wild type.

The evidence suggests that the trp-2e mutants have altered feedback sensitivity in the biosynthesis of tryptophan. Preliminary data show that anthranilate synthetase inhibition by tryptophan is unaffected, and it is therefore probable that the mutation is involved in the activity of PRTase or possible PRA isomerase.

FIGURE 2. Panel A. Intracellular Tryptophan (Micromoles/g dry weight) ●—● Strain 74A; ○—○ Strain 92841-1. Panel B. Induction of Kynureninase (Units/mg dry weight × 10³) ●—● Strain 74A; ○—○ Strain 92841-1. (One unit equals 0.01 Micromoles Anthranilic Acid Produced from Kynurenine/min.)

GENETIC ANALYSIS OF REGULATOR MUTATION TRP-2e IN NEUROSPORA CRASSA

Investigator:

J. R. Turner

Technical Assistance:

Lucille M. Butcher

A mutant of N. crassa has been isolated which exhibits an alteration of the feedback regulation in tryptophan synthesis such that the biosynthetic intermediate phosphoribosyl anthranilate is excreted into the growth medium. This mutation has been mapped on linkage Group IV. It lies close to the marker mtr (4-methyl tryptophan resistance) but is removed from the trp-4 locus (PRTtransferase).

Regulation of the tryptophan biosynthetic pathway in Neurospora crassa by end-product feedback inhibition occurs at two points in the pathway: (1) anthranilate synthetase (AAase) and (2) anthranilate-5-phosphoribosylpyrophosphate phosphoribosyltransferase (PRTase). Feedback insensitive mutants at these points, i.e., regulatory mutants, have not been identified in Neurospora although they have been found in bacteria. We have isolated a series of mutants in Neurospora (tentatively designated trp-2e) which appear to have a defect in feedback inhibition of PRTase. This report describes genetic analysis of this mutation.

OBSERVATIONS

Initial genetic localization of trp-2e was made through crosses with a standard strain designated "alcoy." The alcoy strain contains three chromosomal aberrations which create com-

pound chromosomes such that an unknown mutation can be assigned to no more than two linkage groups on the analysis of four characters within the progeny of a single cross. The results of a cross of trp-2e with alcoy are shown in Table 1. The small number of recombinants of trp-2e with cot (19%) indicates linkage to Groups IV or V. Conversely, the higher percentage recombinants with the other characters al and ylo show that trp-2e is not linked to Groups I, II, III, or VI. Cross of trp-2e to strains containing double and single mutations in Groups I, III, IV, and V were made to confirm the results of alcoy cross. The results of crosses to Groups I, III, and V markers are shown in Table 2. No linkage of trp-2e was found to any of these markers. Linkage to Group IV markers was examined (Table 3) in the progeny of three point cross with trp-4, pan-1, a double mutant in linkage Group IV. Close linkage between trp-2e and both

TABLE 1. Cross 92814-1 (trp-2e) X "Alcoy" Tester

	e +	e -	Total Parental	% of Progeny	Total Recombinants	% of Progeny	Linkage to Group
cot	⁺ 21	59	132	81	32	19	IV, V
	73	11					
al	⁺ 47	37	84	51.2	80	48.8	I, II
	- 47	33					
ylo	⁺ 21	24	50	59.5	34	40.5	III, VI
	26	13					

TABLE 2. Cross 92814-1 (trp-2e) X Group I, III, V Markers

Allele Number	Total Parentals	% of Progeny	Total Recombinants	% of Progeny	Linkage Group Tested
K744, 36104 (hist-1, me-3)	78	48	85	52	V
33933, 37401 (lys-1, inos)	63	42	87	58	V
(eth-1)	95	48.7	100	51.3	I
10575 (trp-1)	88	50	89	50	III

TABLE 3. Three Point Cross 92814-1 (trp-2e) X Y2198, 5531
(trp-4, pan-1) Linkage Group IV

Parentals:	Total Number	% of Progeny	Total Number	% of Progeny
e trp-4 ⁺ pan-1 ⁺	68			
e ⁺ trp-4 pan-1	61	78.6		
Recombinants, Total	35	21.4		
Recombinants Between Single Markers				
e ⁺ trp-4 ⁺	14		e ⁺ pan-1 ⁺	13
e trp-4	7	12.8	e pan-1	22
				21.4
trp-4 ⁺ pan-1	8			
trp-4 pan-1 ⁺	6	8.5		

markers is indicated by the recovery of a small number of recombinants containing **trp-2e** and each of the two known markers **trp-4** and **pan-1**. The **trp-2e** mutation however lies outside the genetic area bounded by these two markers since the map-distance (1 map unit equals 1% recombination) between either of the two markers and **trp-2e** is greater than the distance between **trp-4** (PRTase) and **pan-1**. Since the position of **trp-4**, **pan-1** relative to the centromere is known (centromere--**trp-4**--**pan-1**), the recombination data from the **trp-2e** cross can be used to assign the probable order among these three markers on the chromosome. The results of such a cross suggests the following relative position for the three markers: **centromere--trp-2e--trp-4--pan-1**. This order is further substantiated (Table 4) by the results of a cross with **mtr**, 4-methyl-tryptophan resistance, a marker which lies 8.6 units proximal (toward the

centromere) to **trp-4**. This cross shows that **trp-2e** and **mtr** are within one map unit of each other, thus placing **trp-2e** 8.6 ± 1 map units proximal to **trp-4**.

CONCLUSIONS

In general, a mutation which alters the feedback sensitivity of an enzyme is found within the genetic locus which codes for that enzyme. Such a mutation often results in a structural change in the enzyme which alters the affinity of the enzyme for an inhibitor, usually the end-product of the pathway. The **trp-2e** mutation lies outside of the **trp-4** (PRTase) locus but appears to have some role in its regulation. This characteristic suggests that the mechanism by which regulation is accomplished may involve interaction of a product of the **trp-2e** gene with PRTase or with the **trp-4** locus during transcription.

TABLE 4. Cross 92814-1 (**trp-2e**) X Y30539, r (**ylo-1**, **mtr**)

Parentals:	<u>Total Number</u>	<u>% of Progeny</u>	<u>Linkage Group</u>
e ylo ⁺	194	49.5	VII
e ⁺ ylo	99		
Recombinants			
e ylo	204	50.4	
e ⁺ ylo ⁺	94		
<hr/>			
Parentals:			
e mtr ⁺	399	99	IV
e ⁺ mtr	187		
Recombinants			
e mtr	6	1	
e ⁺ mtr ⁺	0		

THE ROLE OF CALCIUM IN THERMOLYSIN CATALYSIS:
SUBSTITUTION WITH STRONTIUM AND ITS EFFECT ON KINETIC PROPERTIES

Investigators:

*H. Drucker and D. Soderlund**

*When strontium is substituted for calcium as the stabilizing metal in the bacterial **protease** thermolysin, gross changes in the kinetic parameter, K_m , are observed with relatively small changes in V_{max} . The affinity of the enzyme for strontium appears to be 20 times less than the affinity of the enzyme for calcium.*

Calcium is not directly involved in catalytic cleavage of **peptide** bonds by the bacterial **protease** thermolysin. It does appear to affect the secondary structure of the enzyme, preventing autodigestion and inactivation of the enzyme. In addition, changes in calcium concentration appear to cause changes in kinetic properties of the enzyme in cleaving the synthetic substrate 3-2 (furylacryloyl) glycyl-leucinamide (FAGLA). That is, the kinetic parameters K_m and V_{max} vary with calcium concentration, reaching maximum values at a calcium concentration of $10^{-4}M$.

To gain a further insight into the role of stabilizing metals in the chemical and physical properties of this enzyme, strontium was substituted for calcium in the protein, and changes in kinetic properties of the strontium derivative of the enzyme examined.

OBSERVATIONS

Solutions of thermolysin were equilibrated with varying concentrations of strontium chloride by dialysis. The modified enzyme was assayed for activity using the substrate FAGLA. When initial velocity of hydrolysis of this substrate was plotted as a function of $-\log$ strontium concentration (pSr^{2+}), a maximum in initial velocity occurred at pSr^{2+} corresponding to a concentration of $2 \times 10^{-3}M$ (Figure 1). The position of maximal initial velocity with respect to pSr^{2+} did not change with temperature, although activity loss did occur with increasing temperature. Previous studies of this same type employing the calcium derivative of the enzyme showed maxima in the kinetic parameters K_m and V_{max} occurring at $10^{-4}M$ Ca^{2+} . Using these maxima as a measure of the affinity of enzyme for metal, there would appear to be a 20-fold difference in the affinity of enzyme for Ca^{2+} and Sr^{2+} . These data suggest that strontium may be binding

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the same sites as calcium but with decreased efficiency, as previous studies implied that the same sets of calcium binding sites were involved in prevention of autodigestion, activity loss, secondary structure change, and change in kinetic properties of the enzyme.

When kinetic plots of initial velocity versus concentration of substrate are made for enzymes equili-

brated with identical concentration of calcium or strontium, gross qualitative differences in kinetic parameters are observed (Figure 2). K_m and V_{max} determined from such plots are compared in Table 1. The effect of the substitution appears to be primarily on the affinity of the enzyme for substrate. K_m has increased 3.2-fold in the strontium-substituted enzyme whereas the constant expressing catalytic efficiency (V_{max}) remains relatively unchanged. These

FIGURE 1. Effects of Strontium on the Kinetics of Hydrolysis by Thermolysin of the Synthetic Substrate 3-2 (Furylacryloyl) Glycylleucinamide by Thermolysin [Thermolysin at a concentration of 1 mg/ml was dialyzed against the indicated concentrations of pSr²⁺ in 0.1M Tris, pH 8.0 for 12 hr. Enzyme (60 µg) was added to 3 ml of 2.5 mM 3-2 (furylacryloyl) glycylleucinamide in 0.1M Tris, pH 8.0, containing the indicated amount of strontium. Reaction was at 32 °C, and initial velocity was measured spectrophotometrically.]

FIGURE 2. Substrate-Velocity Curves for Thermolysin Equilibrated with Calcium or Strontium Acting on the Substrate 3-2 (Furylacryloyl) Glycylleucinamide [Thermolysin at a concentration of 1 mg/ml was dialyzed against 5 x 10⁻⁴M CaCl₂ or SrCl₂ in 0.1M Tris buffer, pH 8.0 for 12 hr. Enzyme (60 µg) was added to 3 ml of substrate at the indicated concentration in 0.1M Tris buffer, pH 8.0. Reaction was at 32 °C and initial velocity was measured spectrophotometrically.]

data are consistent with the observation that activity of both the calcium- and strontium-containing enzymes are equal at high concentrations of the protein substrate (casein). That is, at protein-substrate saturating levels, equivalent reaction velocities for the two types of enzymes are reached.

TABLE 1. Comparison of Kinetic Properties of Ca^{2+} and Sr^{2+} Equilibrated Thermolysin(a)

Enzyme Equilibrated with	K_m , mM	$V_{\max}$, min ⁻¹
10^{-3}M Ca^{2+}	2.71	0.56
10^{-3}M Sr^{2+}	9.08	0.41

a. Enzyme (1 mg/ml) was dialyzed against 0.1M Tris buffer, pH 8.0 containing the indicated concentration of CaCl_2 or SrCl_2 for 12 hr. The assay mixture contained 60 μg of enzyme, 3-2 (furyl-acryloyl) glycyllleucinamide in 0.1M Tris, pH 8.0 as shown in Figure 2.

CONCLUSIONS

If, as we suggest, Sr^{2+} and Ca^{2+} are bound at the same set of sites, it would appear that Sr^{2+} binding affects the configuration of the protein molecule to the extent that a change in substrate binding sites, as compared to Ca^{2+} occurs, the change is not sufficient to alter the proximity of the amino acid residues involved in catalysis. It is possible that metal replacements of this sort can be used to make a family of enzymes differing in kinetic properties. This would provide a useful tool for studying the effect of small conformational changes on enzymic catalysis.

Studies are presently underway to examine the effect of Ca^{2+} , Sr^{2+} , and other stabilizing metals on the catalytic specificity and tertiary structure of thermolysin.

REGULATION OF EXOCELLULAR PROTEASES IN NEUROSPORA CRASSAI. Induction and Repression of Enzyme Synthesis

Investigator:

H. Drucker

Technical Assistance:

Louise C. Neil

In the presence of a protein carbon source, N. crassa will produce exocellular proteases. The induction is repressed by carbohydrates. The various carbohydrates tested showed differences in their effect on protease biosynthesis. The sugars may be divided into three categories using the effect of the sugar on protease induction as criteria. Carbohydrates in Group 1 repressed synthesis completely. Those in Group 2 had no effect on induction of the exocellular proteases. This lack of effect may be related to the fact that most of the sugars in this group are metabolized with difficulty by cells of N. crassa. The third group is characterized by an acceleration of induction. It is possible that these sugars are not metabolized at all by the cells.

We previously reported that N. crassa will use protein as principal carbon source for growth and that secretion of exocellular proteases parallels growth. We found that sucrose would repress the synthesis of these enzymes and that a material found in culture filtrates of cells growing on 1% bovine serum albumin (BSA) would partially relax the sucrose repression. In this communication, the effect of a wide variety of sugars on protease biosynthesis will be discussed; and further data on the nature of the material relaxing sucrose repression will be presented.

RESULTS AND DISCUSSION

The effect of substrate quantities of carbohydrate carbon sources on pro-

tease production and growth in the presence and absence of BSA (Table 1) varied in a relatively systematic fashion. Easily metabolizable carbon sources, such as glucose or sucrose, repress protease biosynthesis as much as 86%; whereas growth in the presence of BSA was stimulated by 20%. Three sugars (maltose, cellobiose, fructose) supported growth to a lesser extent than sucrose when present as sole carbon source (almost half in the case of maltose); but in the presence of BSA, growth was stimulated from 26-135% as compared to growth on these sugars alone. A concomitant doubling in the amount of protease accompanied growth. A large group of sugars (lactose, mannitol, melibiose, ribose, fucose, arabinose, arabitol) were incapable in themselves of sustaining

TABLE 1. The Effect of Carbon Source on Protease Production

Carbon Source ^(b)	Growth ^(a)		Protease production		Specific Activity	3 Control Protease Production
	- BSA mg/ml Culture	+1% BSA mg/ml Culture	- BSA P.U./ml Culture	+1% BSA P.U./ml Culture	+1% BSA P.U./mg Cells	
Sucrose	3.48	4.14	0	1.202	0.291	14.9
glucose	3.52	4.20	0	1.152	0.274	14.0
D(-)fructose	3.11	3.91	0	1.993	0.511	26.2
D(+)lactose	0	0.412	0	1.164	2.825	145.0
O(-)galactose	0	0	0	0	0	0
D(-)maltose	1.694	3.93	0	1.713	0.431	22.1
D(-)mannitol	0.062	1.35	0	2.162	1.606	82.3
L(-)sorbose	0.028	0.040	0	0	0	0
D(+)cellobiose	1.75	4.18	0	2.175	0.520	26.7
α -D-glucobiose	0.20	0.364	0	1.509	4.147	213.0
D-ribose	0.090	0.863	0	2.035	2.344	120.2
L-fucose	0.034	0.044	0	0.064	1.465	75.1
D-xylose	0.880	1.600	0	0.383	0.242	12.4
D(-)arabinose	0.024	0.450	0	0.562	1.250	64.1
L-arabinose	0.224	1.126	0	2.003	1.779	91.2
L-arabitol	0.056	0.636	0	1.426	2.242	115.0
D(+)arabitol	0.022	0.544	0	1.168	2.147	112.1
Glycerol	0.222	1.252	0	2.566	1.956	102.0
Casamino acids	4.0	4.2	0	0	0	0
Tryptone	4.1	4.3	0	0	0	0
Control-inducing medium	--	2.06	--	4.017	1.950	100.0

a. All parameters were measured at 12 hr of culture.

b. All Media are Vogel's salts + 2% Carbon Source and 1% BSA where indicated.

growth of *Neurospora* but supported in the presence of protein substrate germination and consequently induction of proteolytic activity. Galactose and sorbose were the only sugars that did not support growth, permit germination, or allow protease induction in the presence of protein.

Repression by sucrose is a function of the concentration of the sugar (Figure 1) with 50% repression occurring between 0.7-1% sucrose. As the cells deplete sucrose from the medium containing BSA, synthesis of protease occurs, implying derepression of the protease genes.

FIGURE 1. Effect of Sucrose Concentration on **Protease Biosynthesis** in *N. crassa* Strain 74A [Fifty ml of media containing **Vogel's** salts, 1% BSA, and sucrose in percent (weight/weight) as shown were inoculated with conidia and the culture grown for 16 hr. Samples of culture filtrate were removed and assayed for **protease** activity in the caseinolytic assay (see text). Maximal induction (100% induced **protease** activity) occurs at 0.1% sucrose with **protease** activity corresponding to 2.17 P.U./ml.]

The "inducer" substance for **proteases** was considered previously to be a small molecule on the basis of its thermal stability. Fractionation of this material on Sephadex G-25 demonstrated that a small molecular weight material, void of all **proteolytic** activity, was capable of relaxing sucrose repression and that a large molecular weight material containing active **protease** was not (Figure 2). The inducer substance is not

made when cells are grown under repressing conditions (in 2% sucrose, 1% BSA) and does not appear to be correlated with the action of *N. crassa* **proteases** on BSA. Digestion of protein for periods of 10 min to 8 hr with *N. crassa* **proteases** stripped of factor by Sephadex filtration did not relax sucrose repression.

In a previous report, we examined the kinetics of inducer substance accumulation in cells growing on a

FIGURE 2. Effect of Sephadex G-25 Fractionation on. Activity of "Factor" Produced by *N. crassa* Strain 74A Grown on Inducing Medium (*N. crassa* was grown 8 hr in 100 ml of inducing medium and 5 ml of culture filtrate was removed. This was placed on a 3 × 35 cm column of Sephadex G-25 previously calibrated for void volume with 5 ml of blue dextran. Large molecular weight material, containing all the protease activity in the sample, was eluted in two times void volume. Ten ml of either fraction were added to 100 ml of repressing medium which was then inoculated with conidia of *N. crassa* strain 74A. Samples of culture filtrate were taken at the intervals shown and assayed for proteolytic activity. o—o Proteolytic activity in P.U./ml of filtrate from culture to which large molecular weight fraction was added. x—x Proteolytic activity in P.U./ml of filtrate from culture to which small molecular weight fraction was added.)

medium containing protein as sole carbon source. These observations have been repeated and extended (Figure 3). Inducing substance (factor) is removed from culture filtrates of cells

grown for the times indicated in Figure 3. The material was added to a repressing medium inoculated with conidia. Rate of protease production

FIGURE 3. Time Course of Factor Production by *N. crassa* Strain 74A Growing on Inducing Medium (Conidia were inoculated into 100 ml of inducing medium. At the time intervals shown, 4 ml samples were removed from the culture, and cells were removed by filtration. The 4 ml cell-free samples were added to separate flasks containing 100 ml of repressing medium plus conidia which was subsequently incubated from 0-12 hr. A control culture received no factor. At hourly intervals during the log phase of growth and protease production on these subcultures, samples of culture filtrate were removed and assayed for proteolytic activity. From the data, rates of protease production in units/ml/hr were determined and plotted as a function of time of growth in the original inducing medium.)

was measured in the medium by examining the culture filtrates for protease activity as a function of time. Factor concentration appears to increase in the time interval from 2-6 hr (lag phase to beginning of early log phase), plateaus at 8 hr (early log), and decreases in the interval 8-12 hr (mid-log).

In our previous report, we suggested that trace concentrations of a

metabolite (0.1% sucrose) were essential for protease production with 1% protein as principal carbon source, as the carbohydrate would allow germination and then growth on protein. This report demonstrates that a number of sugars, incapable of supporting growth of *Neurospora*, could trigger germination of conidiospores, permitting growth of the cells on protein as sole carbon source. The nature of

the process which triggers germination is unknown at this time, but should be examined more closely, as it suggests that a general metabolite effect may be responsible for induction of germination. As cells of Neurospora will grow on protein once germination is initiated, there is a possibility of delineating in molecular terms early developmental steps in the transition from conidia to vegetative mycelia.

Inducing substance is not formed by cells growing under repressed conditions. This would imply that it is not an artifact generated by germination in the presence of protein. It is not derived directly from the simple cleavage of protein substrates by mixtures of N. crassa enzymes. Since the enzymes used to arrive at this conclusion were from 8-hr cell cultures, it is possible that factor

is formed by one specific proteolytic enzyme which appeared early in growth and is degraded by proteolytic enzymes synthesized later during growth. This would explain the decrease in amount of factor in mid-log phase of growth. One difficulty with this hypothesis is that under repressed conditions, where little enzyme is made, and where probably the only enzyme produced is the "early enzyme," there is no inducing material made. A further explanation for synthesis of inducing substance would be that it is produced by a sugar-regulated enzyme, not a protease, acting on a product of the early proteolytic enzymes. This possibility is presently under investigation. Preliminary data suggest at least an indirect role for catabolites in the regulation of the early enzyme.

THE REGULATION OF EXOCELLULAR PROTEASES IN NEUROSPORA CRASSA

II. The Role of Specific Proteases in Induction

Investigator:

H. Drucker

Technical Assistance:

Louise C. Neil

Proteases from induced cells of N. crassa, or the bacterial protease thermolysin, can be used to trigger what appears to be the first step in protease synthesis from log-phase cells of N. crassa. The data are consistent with zymogen activation followed by induction of a second protease, the inducer being the zymopeptide produced by zymogen cleavage.

If Neurospora possessed a constitutive ability to produce enzymes capable of degrading protein, there would be little interest in the regulation of these enzymes. As we have shown previously, the ability to produce such enzymes is inducible by a protein substrate and is repressible by a wide variety of easily metabolized carbon sources. This poses a dilemma to the cell as well as a conceptual problem for the investigator. The carbon source which must be used by the cell is a protein which cannot enter the cell. The genetic apparatus coding for synthesis of exocellular protease, on the other hand, is inside the cell. Thus the substrate (protein), unlike other monomeric substrates capable of inducing enzymes, cannot function as an inducer for the exocellular proteases capable of its degradation. In this report, we will discuss the nature of the first steps in **protease** induction, that is, the recognition of external substrate by the cell and the induction of enzymes capable of degrading the substrate.

OBSERVATIONS

Cells of N. crassa, strain 74A, were grown for 12 hr on a medium consisting of Vogel's salts, 1% sucrose. They were then harvested by filtration and suspended in salts for an incubation period of 30 min to deplete carbohydrate reserves. If these cells are then added to a medium consisting of 2% bovine serum albumin plus mineral salts (one part **cells**/one part medium) resulting in a protein con-

centration identical to that found in the medium used to induce **protease** synthesis, only a small amount of **protease** is synthesized over an 8-hr period (Figure 1). If culture fluid from cells induced for **protease** synthesis is added to these log phase cells, there is an immediate production of enzyme (Figure 1). The rate of enzyme production is proportional to the amount of **protease** added to the culture (Figure 2). The material causing this stimulation is judged to be proteolytic enzyme on the basis of heat lability, large molecular weight (as judged by Sephadex G-25 filtration), and the observed correspondence between induction and enzyme concentration.

Three other proteolytic enzymes were tested for their ability to stimulate **protease** production. Equivalent amounts (in terms of **protease** activity) of N. crassa exocellular protease, thermolysin, trypsin, and chymotrypsin were added to log-phase cultures in BSA containing medium. Rates of **protease** production in the culture were measured, and the bacterial **protease** thermolysin was found to be as effective as N. crassa exocellular **protease** in stimulating exocellular **protease** production; whereas trypsin was approximately 1/3 as effective and **chymotrypsin** was without effect (Figure 3). Clearly a specific **protease** is involved in this early step of induction.

The induction of protease, using thermolysin as a stimulator, is followed by growth after a 5-hr time lag (Figure 4). During the lag period,

FIGURE 1. Protease Production of *N. crassa* Strain 74A Transferred in Log-phase from Sucrose to BSA Medium [*N. crassa* was inoculated into 400 ml of medium containing 1% sucrose, Vogel's salts, and cells were grown at 30° for 12 hr. At the end of this time period, the cells were harvested by filtration, suspended in 400 ml Vogel's salts, and starved for 30 min by incubation with aeration at 30°. Twenty-five ml of these cells were transferred to 25 ml medium containing 2% BSA-Vogel's salts, incubated at 30 °C with aeration; and at the intervals shown, 5 ml samples of cells were taken, removed from the medium by filtration, and the cell-free filtrate was assayed for protease activity. ○—○ Protease activity in culture filtrate from culture receiving no additions (control). □—□ Protease activity from culture to which 2 ml of culture filtrate of protease-induced cells were added. The added filtrate contained 3.0 P.U./ml, and protease produced is corrected for this amount of added enzyme.]

FIGURE 2. Protease Production After 4 hr of Log-phase Growth Plotted as a Function of Units of *N. crassa* Exocellular Protease Added (Twenty-five ml of log-phase cells prepared as described in Figure 1 were added to 25 ml 2% BSA-Vogel's salts. *N. crassa* exocellular protease containing 3.0 P.U./ml culture filtrate was added to these cells in amount sufficient to give the indicated amount of proteolytic activity/ml of experimental culture. At 4 hr, 5 ml samples were removed and assayed for protease.)

specific activity of exocellular protease increases, reaching a maximal value at the end of lag. The products of the action of these enzymes on the protein substrate are peptides that can be transported into the cell, resulting in growth that is similar in rate to growth of *N. crassa* on a medium with BSA as principal carbon

FIGURE 3. Substitution of Other Proteases for *N. crassa* Exocellular Protease in Induction of Exocellular Proteolytic Enzyme (One hundred ml of log-phase cells prepared as described in Figure 1 were added to 100 ml of 2% BSA-Vogel's salts. *N. crassa* exocellular enzyme, thermolysin, trypsin, and chymotrypsin were added to cultures at 0 times at a concentration of 0.060 P.U./ml culture. The cells were incubated at 30 °C with aeration; and at the intervals shown, samples of cell filtrate were removed and assayed for proteolytic activity.)

source, and with 0.1% sucrose added to initiate germination of the conidia-spores used as inoculum. The thermolysin-stimulated production of protease from log-phase cells and concomitant growth thus would appear to be similar to the normal induction process.

Rates of induction were found to be dependent on the concentration of thermolysin employed. At low concentrations of the enzyme, induction was seen to be a two-step process (Figure 5A) with the first step of synthesis dependent on enzyme concentration (Figure 5B).

FIGURE 4. Protease Production and Growth in Thermolysin-treated Log-phase Culture (Seven hundred fifty ml of log-phase cells prepared as described in Figure 1 were added to 750 ml 2% BSA-Vogel's salts, and thermolysin was added to a concentration of 0.080 units/ml culture. The cells were incubated as described in Figure 1, and 25 ml samples were removed at the intervals shown. The cells were collected by filtration, lyophilized, and weighed; and culture filtrate was assayed for protease production. $\times$ — $\times$ Growth in mg/100 ml. $\circ$ — $\circ$ Protease in P.U./ml culture filtrate. $\bullet$ — $\bullet$ Specific activity of protease in P.U./mg cells.)

FIGURE 5. Rate of Protease Production as a Function of Concentration of Added Thermolysin (Fifty ml of log-phase cells prepared as described in Figure 1 were added to 50 ml 2% BSA-Vogel's salts, and 0.015-0.120 units of thermolysin were added to the culture prior to incubation. A. Time course of protease production with varying concentrations of added thermolysin. B. Rate of exocellular enzyme production for time 0-3 hr as a function of added thermolysin.)

It is possible that thermolysin stimulates by acting upon protein substrate, resulting in a peptide that is capable of inducing exocellular protease. To test this possibility, an optimal concentration of thermolysin (8 µg/100 ml) was added to a solution of 4% bovine serum albumin plus salts and incubated for time periods of 2 min to 5 hr. This was diluted with salt solution and log-phase cells to

a final concentration of 1% BSA, 2 µg thermolysin/100 ml; and the time course of protease production was determined. If thermolysin acted on the protein substrate, resulting in a product capable of stimulating protease synthesis, then at some point during preincubation of substrate with enzyme this material should be present; and a rate of protease synthesis far greater than that resulting from

addition of 2 μ g thermolysin/100 ml culture should be observed. This was not the case; and the rate of exocellular enzyme production, regardless of period of preincubation, was identical to that expected for 2 μ g thermolysin/100 ml culture.

To examine some of the requirements for induction, cells were incubated with thermolysin without protein for 6 hr (Figure 6A). There is an initial burst of enzyme followed by a gradual decrease of activity. The importance of this decrease will be discussed later. If cells are incubated with thermolysin for time periods of 5 min to 1 hr, washed free of the enzyme, and protein substrate then added, induction does not occur (Figure 6B). The addition of protein substrate to cells for time periods of 5 min to 1 hr, followed by removal of protein and addition of thermolysin does not lead to induction of exocellular protease (Figure 6C). These data imply that thermolysin does not "activate" the cells in some fashion such that subsequent addition of protein causes induction. This would also appear to be the case for protein substrate. Both thermolysin (or *N. crassa* exocellular protease) and protein substrate must be present at the same time to effect induction.

The synthesis of exocellular protease under the conditions employed in this work can be seen from the following experiments to be "induction" rather than large-scale activation of cell-bound enzyme or of intracellular enzyme that has leaked from the cells. Cells were incubated with thermolysin plus BSA, and samples

of cells taken at intervals through a 5-hr incubation period. Addition of thermolysin or *N. crassa* exocellular protease to extracts made from these cells results in no increase in activity (no "activation") (Table 1). If the pellet from the crude extract, containing cell wall, membrane, and cell debris, is treated with thermolysin, again no "activation" of a latent activity is observed.

The possibility of large-scale cell disruption due to treatment with thermolysin with concomitant release of enzyme was examined by electron microscopy. Micrographs of cells at 0 time and after 5 hr of incubation in thermolysin and BSA showed no evidence of gross cell damage.

If the antibiotic cycloheximide (an inhibitor of protein synthesis) is added to cultures of log-phase cells in the presence of thermolysin plus BSA at time intervals from 1-5 hr of incubation, protease biosynthesis stops within 30 min after addition of the antibiotic (Figure 7). These data strongly imply that the production of enzyme is an inductive process in the classical sense of the term.

The above data are negative in the sense that they yield no information concerning the site of action of thermolysin or of *N. crassa* protease in the induction of exocellular proteolytic enzyme. Although freeze-dried cells possessed no activatable component in crude extract or particulate fraction, it was felt that log-phase cells may possess such a material. In the experiments described in Figure 6A, thermolysin was added to cells in the absence of BSA; there was an

FIGURE 6. A. Fifty ml of log-phase cells prepared as described in Figure 1 were added to Vogel's salts containing 0.160 P.U./ml of thermolysin. The cells were incubated as in Figure 1 and protease in culture filtrate assayed. B. Fifty ml of log-phase cells prepared as described in Figure 1 were incubated with 0.160 P.U./ml of thermolysin in Vogel's salts for 1 hr at 30 °C with aeration. The cells were then harvested by filtration and washed with 500 ml of salts to remove added thermolysin. The washed cells were suspended in 100 ml 1%BSA-Vogel's salts and incubated at 30 °C with aeration. At the intervals shown, **exocellular protease** was assayed in culture filtrate. C. Fifty ml of log-phase cells prepared as described in Figure 1 were incubated in 1%BSA-Vogel's salts for 30 min at 30° with aeration. The cells were then harvested by filtration and washed with 500 ml of salts to remove added BSA. The washed cells were suspended in 100 ml Vogel's salts containing 0.15 units/ml thermolysin and incubated at 30° with aeration. At the intervals shown, **exocellular protease** was assayed in the culture filtrate.

immediate increase in protease activity in the culture filtrate followed by a slow decline in activity. It is possible that this could be an "activation" of a protein zymogen yielding an active enzyme. To test this possibility, concentrated cell suspensions of Neurospora were treated

with thermolysin and the increase in amount of protease in the filtrate over that added was measured for 0-30 min. The rate of release of protease from the cells was dependent upon cell concentration and the presence of thermolysin (Figure 8A). This

TABLE 1. Effect of Exocellular Protease of *N. crassa* on Protease Activity of Cell Fractions from Organism^(a)

Fraction	Protease Act. in P.U./ml Fraction	+ Exocellular Protease ^(b)	Sum of Activity Fraction + Added Exocell. Protease	% of Sum Found
Crude extract 0 time	2.575	3.364	5.046	67
Pellet from extraction 0 time	0.272	3.191	2.74	116
Crude extract 5 hr	2.717	4.00	5.188	77
Pellet from extraction 5 hr	0.545	3.414	3.016	113

- a. 250 ml of starved log-phase cells were added to 150 ml 2% BSA. 7.4 P.U. of *N. crassa* exocellular protease were added to the culture; and immediately after this addition, 250 ml of cell slurry were harvested by filtration and Zyophilized. The remaining 250 ml were harvested at 5 hr and Zyophilized.
- b. 2.47 P.U. exocellular protease were added to the fractions.

FIGURE 7. The Effect of Cycloheximide on Thermolysin-Stimulated Induction of Exocellular Protease (Fifty ml of log-phase cells prepared as described in Figure 1 were added to 50 ml of medium containing 2% BSA-Vogel's salts, and 0.2 units/ml culture of thermolysin was added to the medium. o—o At 0 time, cycloheximide was added to one culture to a final concentration of 4 μ g/ml. x—x At 4 hr, cycloheximide was added to a culture at a final concentration of 4 μ g/ml. Exocellular protease was assayed in culture filtrate at the time intervals shown.)

concentration dependence could be described in terms of steady-state kinetics by a Lineweaver-Burke plot which allowed determination of the kinetic parameters K_m and V_{max} (Figure 8B). The data shown are for cells in the presence of cycloheximide + BSA, where no induction (protein synthesis) can occur. The data are identical to those for cell suspensions incubated with thermolysin in the absence of BSA. The implication of these data is that added protease (thermolysin, *N. crassa* exocellular enzyme) acts on some pro-enzyme located on cell wall or membrane (in all probability on membrane) and, by limited proteolysis, converts this inactive enzyme to an active protease. This step, in itself, is not sufficient for induction but could be the activation step (the "first" step) in induction.

FIGURE 8. Rate of Thermolysin-Dependent Activation of Inactive Cell-bound Protease from *N. crassa* (Four liters of 1% sucrose-Vogel's salts were inoculated with conidiospores of *N. crassa* and grown 12 hr at 30° with aeration. The cells were harvested by filtration, suspended in 2% Vogel's salts, and incubated with aeration for 30 min at 30°. The concentrated cell slurry containing 3.75 mg/ml cells was diluted as indicated and 4 μ g/ml suspension of cycloheximide added. 0.121 units/ml suspension of thermolysin was then added and release of protease from cells measured at 5 min intervals for 30 min. Reaction was linear for 15-20 min. A. Rate of protease release in P.U./hr as a function of cell concentration. B. Lineweaver-Burke plot of data.)

The "inducing" component in a system like this must work within the cell at the level of the genes. The question then is: does the action of thermolysin, or *N. crassa* exocellular protease, produce an inducer that is accessible to the inside of the cell? Log-phase cells of *N. crassa* were incubated with thermolysin and BSA (inducing conditions); and at 30-min intervals, over a 5-hr period, samples were removed, and crude extracts made. The protease activity inside the cell is not inducible by protein plus thermolysin (see previous report) and would appear to be constitutive as it is present in the same concentration regardless of growth conditions. Intracellular enzyme produced under any condition of growth is capable of "activating" *N. crassa* cells and stimulating protease production about one-third as well as exocellular enzyme; i.e., three times the number of proteolytic units of intracellular enzyme are required to produce the same rate of exocellular protease synthesis as when thermolysin or *N. crassa* exocellular enzyme is employed. During the course of incubation with thermolysin plus BSA, the specific activity of protease inside the cell remains constant at 1.52 units/mg cell. However, the inducing ability of crude extracts made from cells incubated in BSA-thermolysin medium increases by a factor of two within the first 1-2 hr of incubation, decreases to constitutive levels at 2.5-4 hr of incubation, and remains at this level (Figure 9). Exocellular protease does not change in ability to

FIGURE 9. Effect of Thermolysin Activation on Ability of Induced Cell Extract to Induce Protease (*N. crassa* cells were grown 12 hr in 1 liter 1% sucrose-Vogel's salts and treated as described in Figure 1. One liter of log-phase cells was added to 1 liter 2% BSA-Vogel's salts, and 0.080 units/ml of thermolysin were then added. At 30-min intervals, 100 ml of culture were removed, washed with salts solution and lyophilized overnight. Lyophilized cells were extracted by grinding and concentration of intracellular protease determined (this is constant, varying from 1.5-1.8 P.U./mg cells in activity). Enzyme was added to log-phase cells in 1% BSA-Vogel's salts to an amount equivalent to 0.080 P.U./ml culture, and rate of protease production from these cultures determined.)

induce protease during this time interval. The crude extract "inducing" activity is heat labile and appears to be associated with large molecular weight material.

CONCLUSIONS

From these data there would appear to be at least three steps in the induction of exocellular protease from *N. crassa*. The first step is activation of an inactive proteolytic enzyme by action of protease. Under normal circumstances, this step may be autocatalytic. The inactive enzyme would appear to be constitutive as it is found in sucrose-grown cells. Under the conditions employed here, added enzyme is needed to initiate this step, as the cells are starved and may not be capable of autoactivation of the protease. Evidence for this is that after long periods of incubation, even without added protease, starved cells will produce small amounts of enzyme when incubated with protein. Finally, the activation step is not dependent upon protein synthesis.

The second step appears to be dependent upon protein synthesis as well as concentration of added protease, i.e., the rate of induction varies with concentration of added enzyme. This step is completely inhibited by cycloheximide and therefore may be the classical induction phase.

The third step is not dependent upon concentration of added protease but requires steps one and two. It appears to be classical induction of enzyme.

A speculative model explaining these phenomena can be suggested. Added **protease** hydrolyzes an inactive zymogen. In the presence of protein substrate, the zymopeptide resulting from this activation enters the cell and serves as the inducer. These two steps reflect the enzyme-dependent steps of **protease** induction. The third step, which is not dependent upon thermolysin concentration, may be caused by a product of the digestion of the BSA substrate by the enzyme (or enzymes) produced in the early phases of induction. Another possibility is that the third step of induction requires some critical concentration of zymopeptide. When the correct concentration of zymopeptide is reached, **protease** synthesis proceeds. The rate of this third step would not be a function of thermolysin concentration, but the time of its occurrence would be.

The experiments describing use of crude extract in **protease** induction are in keeping with the suggested model on some points. The inducing ability of extract is not correlated with the amount of proteolytic activity in the extract. The increase in inducing activity of the extract occurs during the second (thermolysin-dependent) step of **protease** synthesis and decreases during the third step. However, the primary effect on induction by crude extract appears to be at the level of the third step of enzyme synthesis. (In Figure 9, rates are shown for 2.5-5 hr of incubation, which is the time of onset of the third step of synthesis.) Resolution of this problem requires an analysis of crude extract components and a separation of ability to induce from ability to activate cell-bound inactive **protease**. These studies are presently underway.

METABOLISM AND EFFECTS OF RADIONUCLIDES IN THE DEVELOPING FETUS
AND THE YOUNG

Many of the biological parameters used to calculate allowable concentrations and permissible exposure levels for radioactive materials are inappropriate for the rapidly growing infant or child or for the pregnant individual. These differences, together with the greater intrinsic radiosensitivity of the immature organism, emphasize the need for more detailed information on the metabolism and toxicity of radionuclides in the premature and juvenile animal. The studies reported here have been designed to help provide these data.

Progress reports are included this year on long-term studies of the effects of exposure to ^{131}I and ^{239}Pu , as influenced by age at time of exposure. Several reports are concerned with the metabolism and effects of einsteinium and berkelium, elements 99 and 97, which have only recently become available in quantities sufficient for biological study. Further reports on these two radionuclides are included in the section on Removal of Internal Emitters.

Our work on radioelements in rodents was particularly benefited during 1971 by the macroautoradiographic skills of Dr. Masatoshi Kashima, a visiting scientist from the National Institute of Radiological Sciences, Chiba, Japan.

THE CARCINOGENICITY OF ^{131}I RELATIVE TO AGE AT EXPOSURE

Investigators

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Groups of prenatal, newborn, weanling, and adult rats were exposed to multiple graded doses of ^{131}I and followed until death or sacrifice at 30 months of age. After examination of about one-half of the histologic sections, it appears that the relationships between the incidence of thyroid tumors and dose are a function of age at the time of exposure. A number of control animals of the newborn and prenatal groups but not of the adult and weanling groups developed thyroid tumors.

Studies in several strains of rats have demonstrated that administration of appropriate dose levels of ^{131}I leads to an increased incidence of tumors of the thyroid gland. There are indications from retrospective epidemiologic studies that juvenile humans are more susceptible to the carcinogenic action of ^{131}I than are adults. Because of the limitations of such studies, we have undertaken a quantitative comparison of the sensitivity to neoplastic changes in the thyroid gland relative to age at the time of exposure to ^{131}I in the rat.

OBSERVATIONS

As described previously (BNWL-1306), prenatal, newborn, weanling, and adult rats were exposed on 5 consecutive days to one of three levels of ^{131}I . The adult and weanling animals were

injected intragastrically;* the newborn animals were exposed via the milk by gavage of the dam on the day of parturition and on the next 4 days; the prenatal animals were exposed by gavage of the pregnant animal during the last 5 days of gestation. The adult animals and the mothers of the animals of the prenatal and newborn groups received 30 (high group), 3.0 (medium group), or 0.2 $\mu\text{Ci/day}$ (low group). The corresponding groups of weanlings received 7.5, 0.75, or 0.05 $\mu\text{Ci/day}$. Each age-dose group consisted of approximately 50 rats of each sex. Groups of control animals were exposed to an identical solution except that ^{131}I was not present. The experimental design anticipated pooling of the control groups; the size of the control groups for

* BNWL-1306 erroneously states "intraperitoneally."

each age was planned to be approximately one-fourth of the size of the experimental groups. Fortuitously, additional animals were available at the time of exposure so that the control groups were larger than originally planned.

On the basis of preliminary experiments, dose levels were selected which would deliver the same range of radiation doses to the thyroid of all age groups. A limited number of animals were sacrificed throughout and following the exposure period and the ^{131}I content of the thyroid gland determined for calculation of radiation doses which were found to be in the range predicted. Since the effects observed in these experi-

ments are so markedly dose-dependent, the dosimetry experiments are presently being repeated and extended. This will provide a more solid basis for relating tumor incidence to radiation dose as a function of age, time, and administered dose.

Necropsies were performed on all animals at the time of death or at 30 months of age. The thyroid and pituitary glands were examined grossly and skip-serial histologic sections prepared. The experiment was initiated in several increments; the time of death or sacrifice likewise extended over a period of several months. Approximately half of the glands have been sectioned and examined as of 1/29/72 (Table 1).

TABLE 1. Effect of Age on the Incidence of Thyroid and Pituitary Tumors in Rats Exposed to Multiple Doses of ^{131}I (Interim tabulation - 1/29/72)

Age-Group	Dose	Thyroid				Pituitary	
		No.	CA	AD	Combined Percent	No.	AD+CA
Adult	Control	8	0	0	0	5	1
	Low	24	1	6	29	21	3
	Medium	20	0	2	10	19	2
	High	17	0	0	0	18	1
Weanling	Control	19	0	0	0	10	0
	Low	29	0	3	10	29	2
	Medium	29	5	13	62	35	4
	High	44	2	6	18	41	4
Newborn	Control	10	0	3	30	20	1
	Low	20	0	3	15	20	1
	Medium	33	0	7	21	25	2
	High	22	0	0	0	19	1
Prenatal	Control	33	2	5	21	33	3
	Low	22	0	5	23	19	0
	Medium	24	0	8	33	25	3
	High	29	3	10	45	25	0

The incidence of thyroid tumors (adenomas and carcinomas) was markedly elevated in the adult animals exposed to the low dose, with a lesser incidence in the medium group, and no tumors in the high group. In the weanlings, tumor incidence was only slightly (10%) increased in the low group but increased to 62% in the medium group and fell to 18% in the high group. The incidences in the medium and high groups were significantly different from the controls.

There were a number of thyroid tumors in the control animals of the newborn and prenatal groups, but not of the adult and weanling groups. Assuming that this difference relates to some factor introduced by manipulation of the dam or the prenatal animals, we find it valuable to confirm this result and to determine the nature of this tumorigenic stimulus.

Because of the elevated control incidence, even the substantial incidence of tumors among the newborn animals exposed to the low and medium

doses did not differ significantly from the controls. The decreased incidence in the rats exposed to the high dose was, however, statistically significant. Likewise, in the prenatally exposed animals, the incidence in the low and medium groups did not differ from the control incidence. On the other hand, those which received the high dose showed a statistically significant elevation in incidence relative to the control.

It may be premature to consider the incidence of carcinoma and adenoma separately since (1) the total number of tumors found is not great and (2) a secondary independent evaluation of the histologic sections has not yet been performed. In general, however, it appears that carcinomas appear predominately in those groups with the highest incidence of adenomas. Marked gross and histologic pathology was noted in several of the hypophyses from animals of all age-dose groups. Neither this nor the incidence of pituitary tumors presently appears to be related to the exposure to ¹³¹I

INFLUENCE OF AGE ON THE LATE EFFECTS
OF MONOMERIC ^{239}Pu IN THE RAT

Investigators:

D. D. Mahlum and M. R. Sikov

Technical Assistance:

Joan O. Hess

The protocol for a study of the influence of age on the oncogenic effects of ^{239}Pu is described. The study has been initiated but no results are yet available.

Studies in other laboratories have shown that ^{239}Pu can be extremely tumorigenic for small animals; osteogenic sarcomas appear to be the major type of tumor induced. In larger animals, induction of other tumors such as hepatomas seem to also be of importance.

Our previous studies with larger doses of ^{239}Pu have demonstrated marked age-related differences in the acute and subacute response to this nuclide. We have shown that the metabolism of the monomeric and polymeric forms varies with age; the LD_{50} values are different for the newborn, weanling, and adult rats; and these relationships differ for the two forms. Our data indicate that there may be a greater risk of death to animals surviving the acute phases of toxicity. Skeletal changes have been more marked in the weanling animal than in the animals of other ages on an administered dose basis. In addition, our previous data indicate that

an increased incidence of mammary tumors may be found in animals treated with ^{239}Pu .

These earlier studies were designed to examine events occurring during the first 9 months after injection of doses of 6 to 60 Ci/kg. No animals were allowed to live beyond 9 months and, therefore, did not attain an age sufficient to allow the observation of osteogenic sarcomas as found by other investigators. Studies performed in other laboratories have not examined the skeletal tumor response as related to age at time of administration. It, therefore, seemed desirable to obtain information on the late effects and on the eventual tumor response, following exposure of animals to ^{239}Pu at several periods during life.

EXPERIMENTAL PROTOCOL

Adult, weanling, newborn, and 19-day fetuses were exposed to monomeric

^{239}Pu by a single intravenous or intracardiac injection or by intravenous injection of the dam. The dose levels (Table 1) were selected to give the same average radiation doses (7, 23, and 70 rads) to the femur in the first 10 postexposure days. These doses also give similar 45-day radiation doses (25, 75, 250 rads) to the three older age groups, but the prenatally exposed animals receive almost no further exposure after the initial 10 days. Rats from each group are being sacrificed at intervals for radioanalysis and autoradiography to establish the microdosimetry for the several organs of importance (components of the skeleton, liver, spleen).

To provide a means for selecting numbers of animals per group, we arbitrarily predicted tumor incidence, based on a general finding of about 50% tumor incidence in adult mice that received $3\ \mu\text{Ci/kg}$. Assuming that arcs in transformation followed by an analysis of variance would be used for data analysis, we selected 50 rats as the group size that would permit detection of sex differences. The experiment was initiated as several replicates begun over a period of more than a year. The statistical analysis technique which we anticipate using will allow for adding additional animals where necessary. Approximately 25 rats of each sex have been exposed in each age-dose group. The prenatally exposed animals were kept

TABLE 1. Doses of Monomeric ^{239}Pu Injected into Prenatal, Newborn, Weanling, and Adult Rats for Determination of Long-Term Effects

Age at Injection	$\mu\text{Ci } ^{239}\text{Pu/kg}$ of Body Weight		
	Low	Med.	High
Prenatal (dose to dam)	6.0	20.0	60.0
Newborn	3.0	10.0	30.0
Weanling	0.3	1.0	3.0
Adult	0.3	1.0	3.0

with their mother until weaning; an additional group of prenatally exposed rats were injected with the highest dose and cross-fostered with untreated litters.

These rats are housed six to eight per cage and are periodically weighed and examined. Moribund animals will be sacrificed prior to death; survivors will be killed at 24 months of age. Radiography and necropsy will be performed at death or sacrifice and organ weights obtained. Organs showing gross pathology will be studied microscopically. Randomly selected rats surviving to 24 months will be subjected to a measure of homeostatic integrity, such as the ability to perform under conditions of cold stress; liver function will be determined in others. It is assumed that clinical examination will suggest other alterations to be quantitated prior to sacrifice.

PLUTONIUM AND THE LIVER OF THE IMMATURE RAT**Investigators:***M. Kashima,* D. D. Mahlum, and M. R. Sikov***Technical Assistance:***Joan O. Hess*

Both parenchymal cell function and reticuloendothelial function of the liver were studied 3 weeks after the administration of monomeric or polymeric ^{239}Pu to newborn, week-old, weanling, or adult rats. The adult and weanling groups showed little effect of the ^{239}Pu on these parameters. Marked impairment of both parenchymal cell and reticuloendothelial function was found in those injected at birth or at 1 week of age, especially after treatment with monomer.

We have shown that the distribution of injected Pu, including the influence of physico-chemical form on its partition between liver and bone, changes relative to age at the time of administration.⁽¹⁾ The resulting biological effects during the interval between 1 and 9 months postinjection were found to be similarly age-dependent.^(1,2) The present study was undertaken to evaluate changes occurring at earlier times after administration.

OBSERVATIONS

Rats of the Charles River CD strain were injected with either monomeric or polymeric ^{239}Pu at 0, 7, 21, or 100 to 140 days of age. The plutonium was injected into the tail vein of the two older age groups and into

the ventricle of the heart in the two younger groups. Doses were chosen, based on previous studies, to produce minimal mortality in a 21-day period. These doses (60 $\mu\text{Ci/kg}$ for monomer and 30 $\mu\text{Ci/kg}$ for polymer) were in the same ratio as the monomer/polymer LD_{50} 's for both the adults and weanlings.

Three animals from each group were sacrificed at 2 and 21 days postinjection for preparation of macroautoradiographs.⁽³⁾ At 21 days postexposure, six rats of each group were intravenously injected with ^{198}Au colloid and sacrificed 10 min later for determination of the function of the reticuloendothelial portion of the liver. Aliquots of blood, as well as the liver, spleen, and one femur, were removed, weighed, and the ^{198}Au content determined by gamma scintillation counting.

An additional six rats from each treatment group were intravenously

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injected with ^{131}I -rose bengal for evaluation of the function of the parenchymal portion of the liver. Samples of blood from the tail were collected in capillary tubes at intervals after injection of rose-bengal and the radioactivity determined by gamma-scintillation counting. The concentration in the blood was determined and the time required for clearance of 50% of the dose calculated.

The partition of Pu, as visualized autoradiographically, was similar in the weanling and adult rats to that reported for the mouse;(4,5) the polymer was deposited predominantly in the liver and spleen, and the monomer in bone. The polymer was seen to localize into discrete foci in the

liver, while the monomer was more uniformly distributed (Figure 1, A & B). After injection of the monomer, the activity was predominantly in the osseous portion of the femur, while the polymer was mainly located in the marrow. The differences between the distribution of the two forms was much less marked in the newborn and 7-day-old rats. Moreover, the polymer was less discretely localized into foci in the liver than in the older animals (Figure 1, C & D). It was also found that autoradiographs of newborn rats exposed to the polymer consistently showed darkening over the heart; this was not seen in those exposed to the monomer.

FIGURE 1. Autoradiographs from Rats Sacrificed 2 Days After Injection with 60 $\mu\text{Ci/kg}$ Monomeric (Upper) or 30 $\mu\text{Ci/kg}$ Polymeric ^{239}Pu (Lower) [The liver, spleen, and femur are shown from left to right for animals injected as adults (A), weanlings (B), and when 1-week old (C). Whole-body autoradiographs of animals injected as newborns are shown in (D).]

These exposures to Pu produced little effect on the gross appearance or relative weight of the liver in the weanlings or adults. In the newborn and 7-day-old animals, there was a disproportionate reduction in liver weight relative to body weight, as well as an inhibition of overall body growth (Table 1). Although both physico-chemical forms produced a marked effect, the newborn rats exposed to the monomer were affected more than those that received the polymer. In the 7-day-old animals, the polymer produced only a slight effect on the weight of the liver, although the monomer was markedly effective. The absolute weights of the spleen and femur were not altered by the administration of Pu and, in fact, were increased relative to body weight,

apparently as a result of the decrease produced in other organ systems.

The phagocytic function of the liver, as measured by uptake of colloidal ^{198}Au , was largely unaffected at 3 weeks after the administration of Pu to adult or weanling animals (Table 2). In the 7-day-old animals, the polymer produced only a slight decrease in phagocytic capacity, although the monomer produced a marked decrease. In the animals exposed at birth, the polymer reduced the uptake of gold colloid to less than half that of the controls; this decrease in function was even more pronounced in those that received monomer. This decreased removal by the liver resulted in a prolonged retention in the blood, which led to an increased uptake by other organs, such as the spleen and femur.

TABLE 1. Effect of ^{239}Pu on Body and Tissue Weight of Rats 3 Weeks Postinjection (Average values $\pm$ Std. Error)

Age at Injection	Form of Pu Injected ^(a)	No. of Rats	Body Weight, g	% of Body Weight		
				Liver	Spleen	Femur
Newborn	Control	6	62 $\pm$ 3	3.96 $\pm$ 0.17	0.48 $\pm$ 0.03	0.36 $\pm$ 0.01
	Monomer	5	51 $\pm$ 1	1.68 $\pm$ 0.003	0.95 $\pm$ 0.04	0.46 $\pm$ 0.01
	Polymer	6	55 $\pm$ 1	2.49 $\pm$ 0.12	0.73 $\pm$ 0.05	0.42 $\pm$ 0.02
One Week	Control	3	118 $\pm$ 3	4.61 $\pm$ 0.08	0.34 $\pm$ 0.02	0.35 $\pm$ 0.01
	Monomer	3	74 $\pm$ 4	2.85 $\pm$ 0.12	0.54 $\pm$ 0.03	0.44 $\pm$ 0.04
	Polymer	3	101 $\pm$ 6	4.15 $\pm$ 0.11	0.28 $\pm$ 0.02	0.37 $\pm$ 0.01
Weanling	Control	5	182 $\pm$ 14	4.66 $\pm$ 0.08	0.27 $\pm$ 0.01	0.32 $\pm$ 0.02
	Monomer	6	135 $\pm$ 8	4.57 $\pm$ 0.07	0.46 $\pm$ 0.04	0.35 $\pm$ 0.02
	Polymer	6	174 $\pm$ 16	4.48 $\pm$ 0.15	0.13 $\pm$ 0.02	0.33 $\pm$ 0.01
Adult	Control	5	299 $\pm$ 17	3.21 $\pm$ 0.05	0.19 $\pm$ 0.01	0.31 $\pm$ 0.01
	Monomer	5	252 $\pm$ 6	3.63 $\pm$ 0.11	0.19 $\pm$ 0.02	0.33 $\pm$ 0.01
	Polymer	5	259 $\pm$ 6	3.67 $\pm$ 0.13	0.07 $\pm$ 0.01	0.34 $\pm$ 0.01

a. Monomer: 60 $\mu\text{Ci/kg}$ injected intracardially (newborn and 1-week rats) or intravenously.
Polymer: 30 $\mu\text{Ci/kg}$ injected intracardially (newborn and 1-week rats) or intravenously.

TABLE 2. Effect of ^{239}Pu Administered 3 Weeks Previously on the Tissue Uptake of ^{198}Au at 10 Min Postinjection (Average values from six rats $\pm$ Std. Error)

Age at Pu Injection	Form of Pu Injected(a)	Percent of Injected ^{198}Au		
		in Liver	per ml Blood	in Spleen
Newborn	Control	91.9 $\pm$ 2.9	0.84 $\pm$ 0.12	1.35 $\pm$ 0.07
	Monomer	2.3 $\pm$ 0.34	20.92 $\pm$ 0.80	7.42 $\pm$ 1.07
	Polymer	40.3 $\pm$ 5.8	10.67 $\pm$ 1.35	5.86 $\pm$ 0.66
One Week	Control	94.5 $\pm$ 2.46	0.19 $\pm$ 0.033	1.19 $\pm$ 0.12
	Monomer	34.8 $\pm$ 5.17	7.41 $\pm$ 0.810	8.10 $\pm$ 1.16
	Polymer	81.9 $\pm$ 9.87	0.10 $\pm$ 0.04	1.13 $\pm$ 0.46
Weanling	Control	94.0 $\pm$ 4.47	0.072 $\pm$ 0.014	1.16 $\pm$ 0.14
	Monomer	91.9 $\pm$ 1.72	0.093 $\pm$ 0.018	2.29 $\pm$ 0.12
	Polymer	93.0 $\pm$ 2.98	0.050 $\pm$ 0.004	0.88 $\pm$ 0.10
Adult	Control	91.1 $\pm$ 3.26	0.056 $\pm$ 0.012	1.40 $\pm$ 0.19
	Monomer	94.9 $\pm$ 1.71	0.080 $\pm$ 0.026	1.00 $\pm$ 0.11
	Polymer	92.9 $\pm$ 2.48	0.060 $\pm$ 0.006	0.86 $\pm$ 0.13

a. Monomer: 60 $\mu\text{Ci/kg}$ injected intracardially (newborn and 1-week rats) or intravenously.

Polymer: 30 $\mu\text{Ci/kg}$ injected intracardially (newborn and 1-week rats) or intravenously.

The administration of ^{239}Pu also altered the parenchymal cell function of the liver of the two younger age groups (Figure 2). The monomer was more effective in decreasing this function than was the polymer, as measured by removal of ^{131}I -rose bengal from the blood. No effect was noted in the weanling and only a slight effect, if any, in the adult animals.

CONCLUSIONS

The differing effects produced by monomeric and polymeric Pu are largely explained by differences in radiation dose to the liver. Our earlier studies,⁽⁵⁾ conducted at 9 months following exposure to plutonium, showed a different pattern of results. New-

borns, exposed to these same doses, showed a lesser deficit in liver function while the weanling and adult animals showed a more marked impairment of liver function than that found at 3 weeks in the present study. To a large extent, these differences may be explained on the basis of the time distribution of radiation dose received by the liver. In the adult, and to a lesser extent in the weanling animals, the ^{239}Pu remains in the liver and continues to deliver radiation over a prolonged period of time. In contrast, although the initial dose rate is high in the newborn animals, the Pu redistributes rapidly and is further diluted as a result of continuing growth. This effectively reduces the dose rate so that the liver of newborn

FIGURE 2. Effect of Plutonium on the Hepatic Parenchyma, as Measured at 3 Weeks Following Plutonium Injection by the Clearance of ^{131}I -Rose Bengal from the Blood: □, Control; ○, Monomer (60 $\mu\text{Ci/kg}$); X, Polymer (30 $\mu\text{Ci/kg}$)

animals receives little radiation exposure subsequent to 21 days postexposure.

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DISTRIBUTION AND SUBACUTE TOXICITY OF ^{253}Es IN
WEANLING AND ADULT RATS

Investigators:

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Technical Assistance:

Joan O. Hess

The highest concentration of ^{253}Es was found in the skeleton of the rat whether it was administered to newborns, weanlings, or adults; substantial concentrations were also found in the liver, spleen and kidney. Mortality was greater among adults treated with 50 to 150 $\mu\text{Ci/kg}$ doses of ^{253}Es , but skeletal defects were more prevalent and severe among animals treated as weanlings.

Studies in our laboratory have shown that the metabolism and effect of long-lived, bone/liver-seeking radionuclides, such as Pu and Ce, are related to the age of the animal at the time of administration. The availability of substantial quantities of the short half-lived (20 days) nuclide ^{253}Es provided an opportunity to study the effects of a semi-acute dose of alpha radiation. We have, therefore, examined the distribution of ^{253}Es in newborn, weanling, and adult rats to determine if age-related differences in metabolism are also found with this heavy metal and have examined the toxicity in the two older age groups.

OBSERVATIONS

Weanling and adult rats were injected via the tail vein with a tracer dose of ^{253}Es as the chloride at pH 2; newborn animals were injected intracardially. Animals from each age group were sacrificed at 1, 7, or 30 days postinjection and selected tissues removed and weighed. The ^{253}Es content was determined by direct gamma scintillation counting of tissue aliquots or liquid scintillation counting of ashed tissues.

Additional weanling and adult rats were injected with doses of up to 150 $\mu\text{Ci/kg}$ of body weight of a similar solution; controls received identical solution sans nuclide. These animals are being observed for determination of mortality and pathologic changes, including skeletal alteration. Radiographs of representative animals were

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obtained periodically, as well as radiographs of animals with obvious osseous pathology.

Initially, the concentration of ^{253}Es in the femur was greater than in any other organ in all age groups; this was most marked in the weanlings (Table 1). The percent of the dose in the skeleton, calculated from the deposition in the femur, likewise showed the same pattern. The next most important site of deposition in all age groups was the liver. In the animals injected as adults or at birth, the fraction of the dose in the liver decreased with time. Those injected at weaning, on the other hand, had a slightly smaller percent of the dose in the liver initially, but there was little diminution with time.

At the time of compilation (225 days postinjection), the adults exposed to 100 or 150 $\mu\text{Ci/kg}$ and the weanlings exposed to 150 $\mu\text{Ci/kg}$ have shown greater than 50% mortality (Table 2). At this time, there is a significant elevation in mortality in the adults exposed to 50 $\mu\text{Ci/kg}$ while the comparable **weanling** group has experienced no mortality. This increased susceptibility can also be seen from examination of the median survival times.

The weanlings, on the other hand, were more susceptible to the induction of gross lesions of the skeleton than were the adults. In the animals of the younger age groups, this was mostly fracture of long bones often accompanied by abnormal healing. In the

TABLE 1. comparison of the Distribution of ^{253}Es in Rats Injected as Newborns, Weanlings, or Adults (Mean $\pm$ Std. Error)

Age at Injection	Days Post-Injection	No. of Animals	Liver		Spleen		Kidney		Femur	
			% Dose	% Dose/g	% Dose	% Dose/g	% Dose	% Dose/g	% Dose	% Dose/g
Newborn	1	7	11.0 ± 0.63	39.9 ± 2.69	0.28 ± 0.04	14.6 ± 2.51	1.37 ± 0.11	16.4 ± 1.80	0.75 ± 0.11	43.9 ± 2.50
	7	6	7.44 ± 0.88	13.8 ± 1.96	0.18 ± 0.05	1.79 ± 0.60	1.20 ± 0.09	5.26 ± 0.33	1.08 ± 0.02	18.0 ± 1.13
	30	2	5.03 ± 0.57	3.76 ± 0.68	0.50 ± 0.13	4.72 ± 1.70	1.96 ± 0.24	3.58 ± 0.06	1.49 ± 0.11	8.14 ± 0.18
Weanling	1	5	8.07 ± 1.13	3.27 ± 0.38	0.95 ± 0.22	3.85 ± 1.11	1.86 ± 0.03	2.57 ± 0.11	2.94 ± 0.11	11.9 ± 0.39
	7	5	6.70 ± 0.37	1.45 ± 0.12	0.94 ± 0.14	2.98 ± 0.55	1.43 ± 0.02	1.40 ± 0.11	3.00 ± 0.24	8.53 ± 0.72
	30	4	7.10 ± 1.73	0.91 ± 0.35	0.60 ± 0.16	1.24 ± 0.49	0.62 ± 0.08	0.32 ± 0.02	2.60 ± 0.07	5.04 ± 0.32
Adult	1	5	15.3 ± 0.67	1.20 ± 0.10	1.02 ± 0.22	1.70 ± 0.25	2.90 ± 0.18	1.19 ± 0.09	2.00 ± 0.07	2.22 ± 0.06
	7	5	7.66 ± 0.61	0.53 ± 0.04	1.10 ± 0.07	3.73 ± 0.81	1.69 ± 0.17	0.74 ± 0.10	2.03 ± 0.06	2.39 ± 0.09
	30	5	1.77 ± 0.19	0.12 ± 0.01	0.86 ± 0.10	2.15 ± 0.40	0.96 ± 0.06	0.37 ± 0.03	2.02 ± 0.03	1.98 ± 0.06

adults, on the other hand, the skeletal lesion was most often a deformation of long bones without fracture. Although lesions developed earlier in the animals exposed as weanlings, essentially all of the animals exposed to 100 or 150 μCi and about half of those exposed to 50 $\mu\text{Ci/kg}$ showed lesions when radiographed at 7 months after injection.

It was noted during necropsy of the animals dying early that there was a loss of one or both the upper incisors. The surviving animals were, therefore, examined to determine the incidence of this dental lesion (Table 3). A significant fraction of the adults that received 10 $\mu\text{Ci/kg}$ and of the weanlings that received 50 $\mu\text{Ci/kg}$ and greater were found to have impaired

dentition. This consisted either of complete loss of teeth or destruction such that only a small nub remained. It is particularly noticeable among those injected as weanlings with the three larger doses: 50, 100, and 150 $\mu\text{Ci/kg}$.

Examination of whole-body autoradiographs of mice sacrificed at 1 day after injection with ^{253}Es in a concurrent experiment indicated a substantial localization of the nuclide in the region of the tooth buds. Since this particular defect has not been noted in our other studies involving ^{239}Pu or ^{144}Ce , it seems that a more detailed examination of the mechanism for this impaired dentition should be made.

TABLE 2. Comparison of Mortality in Rats Intravenously Injected as Weanlings or Adults with ^{253}Es (No. dead at 7-1/2 months/No. injected)

Age at Injection	Injected Dose ($\mu\text{Ci/kg}$ body weight)				
	0	10	50	100	150
Adult	0/30	1/30	7/20	14/20 (MST (a)=178 days)	10/20 (MST (a)=122 days)
Weanling	0/62	1/30	0/20	3/20	13/20 (MST (a)=193 days)

a. Median survival time

TABLE 3. Incidence of Impaired Dentition^(a) in Rats Injected 7 Months Previously as Weanlings or Adults with ^{253}Es

	Injected Dose ($\mu\text{Ci/kg}$ body weight)					
	0	2	10	50	100	150
Adult	0/30	1/40	11/30	3/15	3/8	--
Weanling	1/62	0/29	0/29	20/20	18/18	11/11

a. Teeth are either missing or extremely small.

One apparent osteogenic sarcoma has been observed in radiographs obtained thus far. It was associated with the scapular area in an animal exposed as a weanling to 50 $\mu\text{Ci/kg}$.

CONCLUSIONS

This experiment is still in progress, but some conclusions are already apparent. The greatest fraction of ^{253}Es injected into the blood stream of newborn, weanling, or adult rats is deposited in the skeleton, with the liver being the second most

important site of deposition. The adult animal is more susceptible to the subacute lethal effects of ^{253}Es . The incidence and severity of skeletal alterations produced by the administration of ^{253}Es is higher in animals injected as weanlings than in those injected as adults. Marked effects on dentition are noted in both weanlings and adults treated with ^{253}Es . This may be related to an early localization of the nuclide in or near the tooth bud. Observations in this experiment will continue.

DISTRIBUTION OF ^{253}Es IN THE FETOPLACENTAL UNIT OF THE RAT

Investigators:

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Technical Assistance:

Joan O. Hess

The cross-placental transfer and distribution of ^{253}Es in the fetoplacental unit of the rat was determined 24 hr after injection at 15 or 19 days of gestation. The fraction entering the fetus was much lower than that observed with other heavy metals previously studied. The concentration in the membranes relative to that in the placenta was likewise much lower although the distribution within the membranes was similar to that previously found with ^{239}Pu .

Previous studies in our laboratory^(1,2) have shown marked similarities, as well as differences, between the cross-placental transfer

of heavy-metal nuclides, such as ^{144}Ce , ^{233}U , ^{237}Np , and ^{239}Pu , and between their distribution throughout the fetoplacental unit (FPU).

The recent availability of ^{253}Es permitted us to extend our studies on the behavior of heavy metals in the FPU to the transplutonic elements.

OBSERVATIONS

Pregnant rats were injected intravenously with ^{253}Es (chloride at pH 2) after 15 or 19 days of gestation and killed 24 hr later. The distribution in the dam and in various portions of the fetoplacental unit was determined by counting and autoradiography. The concentrations (1.3-1.9%/g) in the liver, spleen, and femur of the dam were not markedly dissimilar to one another at the two stages of pregnancy (Table 1).

The average concentration in the fetus was greater when injection was made after 19 than after 15 days of gestation, presumably as a result of

accretion by the fetal skeleton. In both cases, the concentrations of ^{253}Es found in the fetuses (0.002 and 0.008 %/g at 16 and 20 days of gestation, respectively) were very much lower than the values in these tissues of the dam, and were several-fold lower than the concentrations found after the administration of the nuclides previously studied. As was observed with these other materials, the concentration in the fetal liver was much lower than that in the femur. The average concentrations in the placenta and fetal membranes were higher at 19 than at 15 days of gestation but at both times were approximately 100-fold greater than in the fetuses (Table 2). These ratios fell within the range of values observed in previous experiments. The ratios of the concentrations in the fetal membranes to those in the placenta calculated from these data were 1.6 and

TABLE 1. Distribution of ^{253}Es in Various Structures at 24 hr After Intravenous Administration to Pregnant Rats (% of injected dose/g wet weight $\pm$ Std. Error)

<u>Structure</u>	<u>Dam^(a)</u>	<u>15-16 Day Fetus</u>	<u>19-20 Day Fetus</u>
Intact Fetus	--	0.0022 $\pm$ 0.0002	0.008 $\pm$ 0.001
Placenta	--	0.253 $\pm$ 0.024	0.757 $\pm$ 0.049
Membranes	--	0.409 $\pm$ 0.031	0.519 $\pm$ 0.077
Liver	1.22 $\pm$ 0.10	0.012 $\pm$ 0.001	0.011 $\pm$ 0.001
Femur	1.89 $\pm$ 0.09	--	0.148 $\pm$ 0.023
Spleen	1.08 $\pm$ 0.16	--	0.004 $\pm$ 0.002

a. Pooled data from dams injected at both times of gestation.

0.7 at the two times of sacrifice. This is in marked contrast with the ratios observed with the other nuclides which were in the range of 4 to 6 with one exceptional value of 16. From the autoradiographs it appeared that most of the activity in the membranes was associated with the villus visceral splanchnopleure of the yolk sac, as was previously found with ^{239}Pu . At both times of sacrifice, the concentration in the decidua was greater than in the labyrinth of the placenta with high levels of localized activity in the trophoblast.

CONCLUSIONS

The fraction of a dose of ^{253}Es entering the fetus after I.V. injection

into pregnant rats is lower than that observed with several other heavy metals. The concentration of ^{253}Es in the fetal membranes, relative to that in the placenta, was also lower than that observed with these other nuclides. As with ^{239}Pu , most of the activity in the membranes was in the villus visceral splanchnopleure of the yolk sac.

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TABLE 2. Concentration of Nuclides in Placenta and Fetal Membranes Relative to Concentrations in Corresponding Fetuses

Nuclide	Placenta/Fetus		Membranes/Fetus	
	Day of Gestation		Day of Gestation	
	15-16 Days	19-20 Days	15-16 Days	19-20 Days
^{144}Ce	19	22	94	89
^{233}U	1	2	16	11
^{237}Np	23	8	135	52
^{239}Pu	80	140	386	492
^{253}Es	115	95	186	65

^{253}Es AND ^{249}Bk DISTRIBUTION IN RAT TISSUES FOLLOWING
INTRAGASTRIC AND INTRAVENOUS ADMINISTRATION

Investigators:

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Tissue distributions of ^{253}Es and ^{249}Bk were measured following intravenous and intragastric administration of the chloride in young adult male rats. Approximately 4×10^{-3} percent of each element was recovered in body tissues 21 days following intragastric intubation.

Following intravenous injections, gross tissue distributions of two radionuclides were generally similar with highest persistent concentrations in bone, followed by spleen, kidney, and liver. The biological half-life of ^{249}Bk in bone appears to be less than 100 days, unusually short for an actinide. Little loss of ^{253}Es from bone was observed, suggesting a behavior typical of most actinides. Urinary excretion of ^{253}Es was initially very high with nearly 40% of the ingested dose excreted during the first day postinjection.

As the higher atomic number elements become available, it is of considerable interest to determine whether their behavior is consistent with that predicted from the lower actinides. Such information is needed for defining conditions for their use and is valuable in developing an understanding of the behavior of the actinide series. Prior to 1971, no studies had been made of the behavior of ^{253}Es or ^{249}Bk in animal systems.

OBSERVATIONS

One mCi ^{253}Es or 0.5 mCi ^{249}Bk in 2 ml of 0.2 N HCl was administered intragastrically to young adult male rats weighing approximately 400 g. The animals were given food and water

ad libitum before and after isotope administration and sacrificed in groups of seven at 4 and 21 days. Table 1 gives data for femur, liver, and total animal. Whole-body counts of ^{253}Es made at the time of sacrifice indicated 3×10^{-2} and 5×10^{-3} percent retention at 4 and 21 days, respectively. This is in reasonable agreement with the ashed tissue values of Table 1. The observed uptake of both isotopes by the intestinal tract is low and consistent with that of other actinides. All data in this paper are corrected for decay to time of isotope administration.

In intravenous injection studies, young adult male rats of a Wistar-derived strain weighing approximately

TABLE 1. Retention of Intragastrically Administered ^{253}Es and ^{249}Bk (% of administered dose $\times 10^3$, mean values $\pm$ standard deviation.)

	Time Post-Administration	Femur	Liver	Total Body
^{253}Es	4 days	0.39 $\pm$ 0.13	0.56 $\pm$ 0.016	35 $\pm$ 22
	21 days	0.14 $\pm$ 0.05	0.073 $\pm$ 0.021	3.9 $\pm$ 0.78
^{249}Bk	4 days	0.035 $\pm$ 0.019	0.21 $\pm$ 0.11	7.3 $\pm$ 2.5
	21 days	0.71 $\pm$ 0.061	0.023 $\pm$ 0.034	3.4 $\pm$ 5.8

400 g were injected via the tail vein with 2.5 μCi ^{253}Es or ^{249}Bk in 0.5 ml of 0.01 N HCl. The animals to be sacrificed at 21 days were individually housed in metabolism cages and urine and feces collected. The other animals were group caged and all were given feed and water ad libitum. Tissue concentrations of ^{253}Es are shown in Figure 1 and show little change in bone or spleen over 83 days. Data obtained at 1 and 7 days post-injection are inconsistent with those obtained at other times and highly variable; mean values for these days are shown but were ignored in constructing the curves.

Data for ^{249}Bk are similarly graphed in Figure 2. It appears that ^{249}Bk is lost more rapidly from all tissues than is ^{253}Es . Both radio-nuclides are lost more rapidly from liver than from the other three tissues. Of several other tissues sampled at 21 days postinjection, thyroid and lung had tissue concentrations of

Bk and Es as high as liver (0.1% of injected dose/g at that time).

Three skeletons were separated from soft tissues at each of the final two sacrifices. These skeletons contained 29 $\pm$ 3 and 36 $\pm$ 4% of the injected ^{253}Es at 21 and 83 days, respectively; and 41 $\pm$ 0.5 and 9.1 $\pm$ 1.2% of the injected ^{249}Bk at 21 and 120 days, respectively. At all times, the skeletal content was greater than that of the liver. The ratio of total skeletal content to the content of one femur was 18-20. The rapid loss of ^{249}Bk from the skeleton indicates a different behavior from ^{253}Es as well as from other actinides. The excretion patterns of the two elements are also quite different as shown in Figures 3 and 4. Nearly 40% of the ^{253}Es was lost in the urine during the first postinjection day, and urinary excretion continued greater than fecal excretion through 21 days. In contrast, fecal excretion of ^{249}Bk exceeded that in the urine except

FIGURE 1. Tissue Concentrations of ^{253}Es Following Its Intravenous Injection as the Chloride into Young Adult Male Rats (Each point is the mean of seven animals. Vertical bars represent $\pm$ standard deviation.)

during the immediate postinjection period. The rate of excretion of ^{249}Bk declined more slowly than did that of ^{253}Es . This is consistent

with the observed rapid loss of Bk from skeleton and body tissues generally.

FIGURE 2. Tissue concentrations of ^{249}Bk Following Its Intravenous Injection as the Chloride into Young Adult Male Rats (Each point is the mean of seven animals. Vertical bars represent $\pm$ standard deviation.)

CONCLUSIONS

Distribution and excretion of ^{253}Es and ^{249}Bk were generally typical of that exhibited by trivalent actinides. Following the immediate post-injection period, concentrations were highest in bone, followed by spleen,

kidneys, liver, thyroid, and lung, in that order. The biological half-time for ^{249}Bk retention in bone appeared to be less than 90 days; that of ^{253}Es was much longer, and thus more similar in behavior to plutonium.

FIGURE 3. Excretion of ^{253}Es Following Intravenous Injection of the pH 2 Chloride (Each point is the mean of seven animals.)

FIGURE 4. Excretion of ^{249}Bk Following Intravenous Injection of the pH 2 Chloride (Each point is the mean of seven animals.)

EFFECTS OF RADIOSTRONTIUM IN MINIATURE SWINE

Investigation of the biological effects of daily ingestion of ^{90}Sr in miniature swine was initiated in our laboratory in 1958. Miniature swine are being used in these studies because they are omnivores with a mature weight, dietary requirement, gastrointestinal tract, and bone mass similar to man. These favorable physiological factors, plus an estimated life-span in excess of 12 years, make these animals a better stand-in for man than the smaller laboratory animals more commonly employed in radionuclide studies. Approximately 800 female miniature swine, representing three generations, have been exposed to feeding levels of ^{90}Sr ranging from 1 to 3100 $\mu\text{Ci/day}$, while 194 untreated female littermates served as controls.

The older animals in this study are now approaching the limits of their expected life-span and definitive statements concerning life-span effects of chronic feeding of ^{90}Sr can begin to be made. As the number of animals in the chronic study decline, increased effort is being expended in answering corollary questions as to possible mechanisms for explaining the observed effects. The very interesting leukemogenesis studies which have evolved from this project are included in the section that follows "Radiation-Induced Leukemogenesis."

TOXICITY OF CHRONIC ^{90}Sr INGESTION

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The primary causes of mortality in both control and ^{90}Sr -exposed miniature swine between 8 and 12 years of age have been periodontal and uterine pathology.

Previous progress reports have detailed the experimental design and purpose of these studies on the effects of chronic ^{90}Sr ingestion in miniature swine. The original study included the daily feeding of 1, 5, 25, 125, 625, or 3100 μCi . The parent generation was started on ^{90}Sr feeding at 9 months of age and then bred to a control male to produce the F_1 generation, which in turn produced the F_2 pigs. Currently, only animals at 1, 5, and 25 $\mu\text{Ci/day}$ survive from the original study. Because of the high incidence of myeloid neoplasms observed in the 125 $\mu\text{Ci/day}$ group, this dose level was expanded to increase the number of animals for observation and is termed the "leukemic pool." Also, in an attempt to reduce the induction time of these neoplasms, a group of F_1 generation animals was produced at the 250 $\mu\text{Ci/day}$ level.

Currently, there are 78 miniature swine remaining in the chronic ingestion study. These include thirty-two controls, twenty-eight 1- μCi offspring,

eleven 5- μCi offspring, two 25- μCi parent generation sows, and five 25- μCi offspring. In addition, 16 of the 21 F_1 generation leukemic pool animals are alive at 26 or 36 months of age. There are also twelve of twenty-one 250- μCi offspring alive 20 months after birth.

OBSERVATIONSChronic Toxicity Animals

There have been no definitive new developments during the past year in those animals remaining in the chronic toxic effects study. As the animals age beyond about 8 years, determining the cause of death becomes very complex (Table 1). Also, separating radiation effects from aging effects and their interactions becomes tenuous.

One of the major contributions to the cause of death in older miniature swine continues to be extensive uterine pathology. This takes the form of

TABLE 1. Deaths in Chronic ^{90}Sr Study During 1971

$\mu\text{Ci/day}$	No.	Gener- ation(a)	Age at Death (year)	Type of Death(b)	Contribution to Death				
					Teeth	Arthritis	Uterine Pathology	Bone Marrow Pathology	Other
0	1250	F	8.4	E					ONS signs
	1272	P	8.4	D			+++		
	1280	P	8.6	D			++++		
	1281	P	8.5	S					
	1286	F	8.4	E	+++	++	+++		
	1347	P	8.3	D				+++	Multiple myeloma; necrotic hepatitis
	1348	P	8.7	D	+	+	+++		
1	177	F	11.7	E		+	++		Necrotic hepatitis; nephritis;
	559	F	11.4	E	+	+	++		emaciation
	561	F	12.4	E	++++	++	+		Mesenteric tumor and ileal intus-
	651	F	11.0	E		+			susception
5	197	F	12.3	D	+	t	++++		ONS signs
	464	F	11.3	E		++	+	+++	Renal fibrosis; red cell aplasia
	801	F	10.1	E	++		++++	+	
	860	F	10.4	D			+++		Fibrinous peritonitis; pleuritis;
25	395	F	11.5	E	+++	++	+	+	pneumonia
	398	F	12.0	E	+++	+			Emaciation; mandibular osteomyelitis
	872	F	10.6	E	+++		++	+	Emaciation; carcinomatosis
	1168	F	9.3	E	++++			+	Emaciation
	1169	F	9.3	E	++++				Emaciation; purulent sinusitis
	1174	F	8.8	E	++	++		++	Pulmonary granulomatosis; severe
									spondylitis

a. P = Parent, F = Offspring

b. D = Died, E = Euthanasia, S = Planned Sacrifice

either varying degrees of chronic endometritis or the development of massive uterine tumors. These animals usually manifest a progressive deterioration in their physical condition necessitating euthanasia. There is no apparent relationship between ^{90}Sr -feeding level and subsequent development of this syndrome.

Arthritis is a common finding in these older swine but rarely causes incapacitation although most joints of weight-bearing bones may manifest degenerative changes. Ankylosing

spondylitis is also frequently observed. This may involve only one or two vertebrae, or frequently may include most of the thoracic and lumbar vertebrae. It is probably most common in the distal thoracic portions of the vertebral column.

With advancing age, dental defects become an increasingly severe problem and are a major factor necessitating euthanasia. Generally, these lesions involve varying degrees of perialveolar degeneration with impacted hair

and feed, resulting in suppurative processes. There are also many cases in which the occlusal surfaces are worn to the gum line with practically no masticatory ability left. Dental defects appear more commonly in older swine ingesting 25 μCi ^{90}Sr /day than at the lower dose levels. This is not surprising in light of previous findings of a relatively high ^{90}Sr concentration in the periodontal tissue.

As would be expected, the diversity of disorders observed in these older swine produces a wide range of effects on the hematologic and serum biochemistry parameters. These pathologic processes tend to obscure any radiation effects at the lower dose levels as the animals approach the end of their normal life-span.

"Leukemic Pool" Swine

These animals are now 2-3 years of age and should be approaching the peak period for development of hematopoietic neoplasms. Certain of these animals have been subjected to tests which assay their immune response, which will be detailed in a subsequent report. The two deaths that occurred in this group during the past year were the result of severe hemorrhagic enteritis, which was not a direct result of ^{90}Sr feeding.

250- μCi /day Offspring

With the exception of two animals, ^{90}Sr feeding was discontinued to this group at 200 days of age because of

the development of severe thrombocytopenia and leukopenia (Figure 1). By 7 months of age, 6 of 21 of these pigs were dead. At 9 months of age (~ 2 months after cessation of ^{90}Sr feeding), the platelet and leukocyte values had shown considerable recovery and remained essentially on a plateau for an additional 9 months. The recovery in leukocyte values are mainly a reflection of an increase in neutrophils.

FIGURE 1. Average Platelet and Leukocyte Values in Control and 250- μCi /Day Offspring (^{90}Sr feeding was discontinued at 200 days of age except for two animals represented by the solid line or dashes.)

Two of the animals removed from ^{90}Sr ingestion have died during the past year, one of parasitic bile duct occlusion at 0.9 year of age and the other at 1.5 years of a **hematopoietic** neoplasm tentatively diagnosed as multiple myeloma.

The prolonged survival of the two animals that continued to ingest 250 $\mu\text{Ci/day}$ is difficult to explain in light of the early deaths at this feeding level by 7 months of age. Their platelet and leukocyte values remain severely depressed, particularly in one animal (Figure 1). Final evaluation of these two animals must await necropsy and radioanalysis data.

CONCLUSIONS

Diseases apparently associated with the aging process in these miniature swine tend to obscure possible effects of chronic ^{90}Sr ingestion at the **lower dose** levels. The merits of this long-term study then become obvious when it is realized the 25- $\mu\text{Ci/day}$ group attains a body burden ~ 125 times the **permissible** ^{90}Sr body burden for man. As sufficient control animals reach 10-12 years of age, it should be possible, statistically, to either eliminate or incriminate ^{90}Sr effects on specific parameters.

UTERINE-MILK EXPOSURE STUDY OF ^{90}Sr EFFECTS IN MINIATURE SWINE

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Technical Assistance:

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Miniature swine were exposed to ^{90}Sr at various combinations of five different pre- and postnatal periods. Three years later definite effects were observed only in those animals continued on ^{90}Sr ingestion after weaning. It appears, from peripheral blood and bone marrow parameters and accumulated mortality, that animals exposed to ^{90}Sr via milk and feed were more severely affected than animals whose exposure included the in utero period in addition to milk and feed.

This and the subsequent three reports deal with a study designed to determine the effects of ^{90}Sr exposure

at different periods of pre- and postnatal development. Particular attention was to be focused on the

subsequent occurrence of hematoproliferative syndromes. Previous studies had shown that 125 μCi ^{90}Sr /day would produce >50% incidence of these disorders in miniature swine. Therefore, this dose level was selected to be fed the dams during gestation and lactation and also for daily feeding to certain offspring. This study involved litter-switching after synchronization of the estrous cycles of control and 125- μCi ^{90}Sr /day sows to result in parturition of all females within a narrow time period. Five different periods were chosen for ^{90}Sr exposure of the offspring, i.e., in utero only (U); nursing only (M); in utero and nursing only (U+M); nursing and then feeding after being weaned (M+F); and in utero, nursing, and feeding (U+M+F). The experimental design is shown in Table 1. The last two groups at weaning were continued on ^{90}Sr by incremental increases so that at 6 months of age they were receiving 125 μCi ^{90}Sr /day. Palmer, et al.⁽¹⁾ have established an accumula-

tive skeletal dose of ~ 50 rads during in utero exposure at the 125- μCi /day level and a similar accumulated dose during the nursing period. Adult pigs at this level accumulated ~ 11 rads/day to the skeleton.

The animals on this study are now 36 months of age.

OBSERVATIONS

The effects of the various ^{90}Sr exposures on peripheral blood values are shown in Figures 1 and 2. As would be expected, the most obvious effects are in those animals continued on ^{90}Sr after weaning. The neutropenia observed in the M+F animals appears to be more pronounced than in the U+M+F group. The cyclic type neutropenia seen in the M group is of interest since it is not apparent in the other groups and has not been observed in any of the previous ^{90}Sr studies. In general, it appears that animals exposed to ^{90}Sr in utero (U, U+M, and U+M+F) experience a more

TABLE 1. Experimental Design and Mortality of Uterine-Milk Exposure Study

<u>Group</u>	<u>Dam</u>	<u>^{90}Sr Exposure</u>	<u>No. of Animals Initial/Dead at 36 months</u>
U	125 μCi	In utero only	10/1
M	Control	Nursing only	10/0
U+M	125 μCi	In utero and nursing	8/0
M+F	Control	Nursing and feed	10/7
U+M+F	125 μCi	In utero, nursing and feed	9/2

FIGURE 1. Mean Leukocyte and Platelet Values in **Uterine-Milk Exposure Study** (see text for group designations)

FIGURE 2. Mean Neutrophil and Lymphocyte Values in **Uterine Milk Exposure Study** (see text for group designations)

pronounced lymphocyte depression than those exposed after birth (M, M+F). This finding is difficult to explain in light of a previous study that indicated little radiation was received

by the thymic region during the gestation period and presumably not by other extramedullary lymphoid tissue.(1)

Bone marrow was aspirated from the sternum when the animals were 33 months of age. The myeloid/erythroid (M/E) ratio was calculated by eliminating segmented granulocytes in an attempt to preclude the peripheral blood contribution and normalize each sample. The results are shown in Table 2. There is a shift to more immature forms of both myeloid and erythroid elements in those animals still on ^{90}Sr ingestion (M+F and U+M+F) and the effect appears more evident in those that did not receive in utero exposure (M+F). This result would occur from either a maturation defect in a clone of cells which could not develop beyond a specific immature stage or a stimulatory effect so that more committed stem cells were in cycle, or prob-

ably by both mechanisms. The influence of early cell death on this cycle must also be considered.

A comparison of the cumulative mortality between groups is of particular interest (Table 1). Only one animal of those not continued on ^{90}Sr feeding has died. Cause of death in this uterine-exposure only animal was a nonradiation related acute necrotizing pancreatitis. However, seven of ten animals exposed to ^{90}Sr via milk and feeding (M+F) have died whereas only two of nine in the U+M+F group were dead by 36 months of age. This difference in mortality seems too great to be insignificant yet is difficult to explain since the difference in accumulated skeletal ^{90}Sr should be only about 50 rads.

TABLE 2. Myelograms of Uterine-Milk Exposure Study in Miniature Swine 33 Months of Age (Mean $\pm$ S.E.)

	Control (3)	U (9)	M (10)	U+M (8)	M+F (7)	U+M+F (7)
Pronormoblast	0.17 $\pm$ 0.08	0.24 $\pm$ 0.07	0.35 $\pm$ 0.08	0.35 $\pm$ 0.08	0.90 $\pm$ 0.27	0.77 $\pm$ 0.18
Basophilic Normoblast	1.73 $\pm$ 0.41	2.38 $\pm$ 0.33	2.75 $\pm$ 0.46	2.16 $\pm$ 0.44	4.37 $\pm$ 1.78	3.94 $\pm$ 0.95
Polychromat NB	15.9 $\pm$ 2.2	19.8 $\pm$ 1.8	19.9 $\pm$ 1.17	20.6 $\pm$ 1.7	29.4 $\pm$ 6.7	27.6 $\pm$ 3.1
Orthochromat NB	1.47 $\pm$ 0.4	1.87 $\pm$ 0.4	2.60 $\pm$ 0.7	2.21 $\pm$ 0.6	5.11 $\pm$ 1.3	2.66 $\pm$ 0.4
Myeloblast	0.37 $\pm$ 0.2	0.23 $\pm$ 0.05	0.61 $\pm$ 0.3	0.38 $\pm$ 0.1	0.86 $\pm$ 0.3	0.40 $\pm$ 0.1
Promyelocyte	0.99 $\pm$ 0.5	0.87 $\pm$ 0.1	0.83 $\pm$ 0.2	0.89 $\pm$ 0.1	1.84 $\pm$ 0.5	1.13 $\pm$ 0.2
Myelocyte	2.2 $\pm$ 0.5	2.3 $\pm$ 0.2	2.2 $\pm$ 0.3	2.6 $\pm$ 0.2	3.2 $\pm$ 0.9	2.6 $\pm$ 0.4
Metamyelocyte						
Neutrophil	7.6 $\pm$ 1.0	7.6 $\pm$ 0.7	7.1 $\pm$ 0.4	8.3 $\pm$ 0.5	7.8 $\pm$ 1.9	7.8 $\pm$ 1.0
Eosinophil	2.9 $\pm$ 0.2	2.5 $\pm$ 0.4	2.3 $\pm$ 0.3	2.1 $\pm$ 0.3	1.2 $\pm$ 0.3	2.8 $\pm$ 0.5
Basophil	0.27 $\pm$ 0.27	0.43 $\pm$ 0.33	0.15 $\pm$ 0.06	0.13 $\pm$ 0.07	0.04 $\pm$ 0.04	0.09 $\pm$ 0.05
Band Neutrophils	22.8 $\pm$ 0.9	30.2 $\pm$ 1.8	26.6 $\pm$ 1.5	27.3 $\pm$ 1.1	20.0 $\pm$ 4.1	24.3 $\pm$ 2.3
Segmented						
Neutrophils	16.6 $\pm$ 2.7	15.0 $\pm$ 1.4	15.3 $\pm$ 1.1	17.6 $\pm$ 2.1	10.8 $\pm$ 2.3	12.4 $\pm$ 1.8
Eosinophil	1.8 $\pm$ 0.3	1.1 $\pm$ 0.3	1.1 $\pm$ 0.3	1.0 $\pm$ 0.3	0.57 $\pm$ 0.2	0.90 $\pm$ 0.2
Lymphocytes	19.6 $\pm$ 2.0	11.8 $\pm$ 1.4	14.9 $\pm$ 1.4	11.3 $\pm$ 0.6	9.1 $\pm$ 2.6	7.6 $\pm$ 1.3
Monocytes	3.3 $\pm$ 0.2	1.7 $\pm$ 0.3	1.2 $\pm$ 0.2	1.2 $\pm$ 0.3	0.5 $\pm$ 0.3	1.5 $\pm$ 0.5
Mitoses						
Erythroid	0.20 $\pm$ 0.1	0.33 $\pm$ 0.1	0.36 $\pm$ 0.1	0.26 $\pm$ 0.1	1.04 $\pm$ 0.2	0.60 $\pm$ 0.2
Myeloid	0.10 $\pm$ 0.1	0.19 $\pm$ 0.1	0.18 $\pm$ 0.1	0.36 $\pm$ 0.1	0.24 $\pm$ 0.1	0.24 $\pm$ 0.1
M/E Ratio	1.97 $\pm$ 0.2	1.88 $\pm$ 0.2	1.66 $\pm$ 0.2	1.68 $\pm$ 0.1	1.50 $\pm$ 0.7	1.19 $\pm$ 0.2
Mitotic Index	0.005	0.005	0.008	0.010	0.016	0.012

One of the U+M+F animals died at 17.8 months of acute suppurative pneumonia, and the other at 23 months of acute myeloblastic leukemia.

Five of the seven M+F deaths were due to severe thrombocytopenia and resultant hemorrhagic syndrome. One of these five also was severely neutropenic with a total leukocyte count of $800/\text{mm}^3$. Bone marrow examination revealed a complete agranulocytosis and 17% plasmacytoid cells, many with myeloma cell characteristics. Total serum protein was elevated (10.8 gm/100 ml) with the gamma globulin component comprising 4 gm/100 ml. A second animal in this group had a slightly elevated white count for this dose level ($11.6 \times 10^3/\text{mm}^3$) and 9% immature granulocytes in the peripheral blood. An aspirated marrow sample contained 8% myeloblasts, numerous smudge cells, giant band neutrophils, nuclear-cytoplasmic asynchrony, and occasional cells with large, retained nucleoli. Pending results of histopathologic examination, both of these cases are considered hematoproliferative disorders.

Of particular interest is one animal exposed to ^{90}Sr during the in utero period only. This pig had maintained an essentially normal hemogram until 36 months of age at which time 11% basophils were noted in the peripheral blood with a leukocyte count of $20 \times 10^3/\text{mm}^3$. A bone marrow sample obtained 2 months previously was unremarkable except for a slight myeloid shift in the M/E ratio and 4.6% basophils. A second marrow sample obtained shortly after the

basophilia was noted revealed 18% basophils present. Close observation of this animal will be continued.

CONCLUSIONS

No definitive conclusions can be drawn so early in a study such as this particularly with the small number of animals involved. However, certain important trends are apparent. Strontium-90 exposure from conception through lifetime feeding appears to be less toxic to the bone marrow than when the exposure is the same exclusive of the in utero period. The difference in mortality between these groups (U+M+F and M+F) seems too great to be incidental. With the longer survival time, a greater incidence of hematoproliferative syndromes will probably be seen in the U+M+F group, rather than the hemorrhagic syndrome common to the M+F animals. The late effects of in utero ^{90}Sr exposure will be of interest compared with those receiving a similar radiation dose during the nursing period.

The effects observed in this group to date merit consideration of initiating a comparable study in a smaller species so that a larger number of animals could be utilized in each group. Early postnatal effects on the hematopoietic system after the various exposure regimens should be critically evaluated.

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THE CHEMICAL FORMS OF HYDROXYPROLINE
IN PLASMA OF MINIATURE SWINE EXPOSED TO ^{90}Sr

Investigator:

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The plasma levels of protein-bound and peptide-bound hydroxyproline were unaffected by exposure to ^{90}Sr . The amount of free hydroxyproline was lower in two groups of offspring of dams receiving 125 μCi than in controls. One of these groups of animals continued receiving ^{90}Sr after they were weaned while the other group received no ^{90}Sr after weaning.

Deposition of ^{90}Sr in skeletal tissue results in delivery of significant amounts of radiation to the skeleton, including its major protein component, collagen. Collagen turnover is slower than that of many other proteins, but recent studies have shown that collagen proliferates rapidly at sites of wounds, irritations, or tumor growth. Whole-body X-irradiation has demonstrated an interference with the conversion of proline to hydroxyproline and an acceleration of collagen degradation.

Histopathologic examination of bone specimens from swine ingesting 125 μCi or 625 μCi ^{90}Sr daily displayed different degrees of fibroplasia and, in many cases, a filling in of the marrow cavity with mature collagen. To initiate an investigation of the effect of ^{90}Sr irradiation on collagen metabolism, plasma hydroxyproline levels were determined in swine on the uterine-milk exposure study.

OBSERVATIONS

Blood plasma samples were obtained from selected animals on the uterine-milk exposure study described elsewhere in this report. The samples were immediately processed to separate the hydroxyproline into three components; total, protein-bound, and free hydroxyproline. The assays were performed according to the procedures of LeRoy, et al.⁽¹⁾ and Kivirikko, et al.⁽²⁾ Table 1 presents data obtained from animals at 30 months of age. There were no statistically significant differences among the various ^{90}Sr treatments, but there was a trend toward a reduction in the peptide-bound hydroxyproline fraction in the groups ingesting 125 μCi ^{90}Sr in the milk plus feeding group and in the uterine plus milk plus feeding group.

Values for the peptide-bound fraction were determined by subtracting the protein-bound and free fractions from the total plasma hydroxyproline.

Since the peptide-bound portion is a relatively small fraction of the total amount, there is considerable uncertainty in these values. To increase the reliability of the peptide-bound values, this reaction was determined in the next sampling, at 36 months, by subtracting the free hydroxyproline from a measure of the total nonprecipitable hydroxyproline. The animals used as controls in this

assay ranged in age from 18 to 24 months. Table 2 shows the result of this assay. There appeared to be no effect on the levels of protein-bound hydroxyproline for any of the treatments, including the controls. The peptide-bound hydroxyproline values were quite uniform and, in general, lower than in the first assay (Table 1). There was a statistically significant reduction in the

TABLE 1. Distribution of Plasma Hydroxyproline in 10-Month-Old Swine Exposed to ^{90}Sr

^{90}Sr Treatment	Plasma Hydroxyproline, $\mu\text{g/ml}$		
	Protein	Peptide	Free
Milk	$13.7 \pm 1.3^{(a)}$	1.96 ± 0.88	6.27 ± 1.04
Uterine	13.7 ± 1.0	2.28 ± 0.95	5.84 ± 1.48
Uterine + Milk	11.2 ± 0.9	3.36 ± 1.63	4.98 ± 0.99
Milk + Feeding	13.6 ± 3.5	0.17 ± 1.48	5.03 ± 1.87
Uterine + Milk + Feeding	12.5 ± 1.6	0.90 ± 1.43	5.89 ± 1.40

a. 90% Confidence Limit of Mean

TABLE 2. Distribution of Plasma Hydroxyproline in 36-Month-Old Swine Exposed to ^{90}Sr

^{90}Sr Treatment	Plasma Hydroxyproline, $\mu\text{g/ml}$		
	Protein	Peptide	Free
None	$13.4 \pm 3.6^{(a)}$	1.37 ± 0.47	8.04 ± 1.96
Milk	14.6 ± 2.3	1.34 ± 0.32	6.51 ± 1.17
Uterine	15.7 ± 1.6	1.35 ± 0.38	4.71 ± 1.40
Uterine + Milk	13.8 ± 1.0	1.24 ± 0.25	3.51 ± 0.42
Milk + Feeding	13.1 ± 3.3	1.04 ± 0.45	5.16 ± 1.22
Uterine + Milk + Feeding	14.3 ± 2.1	1.08 ± 0.31	3.55 ± 1.27

a. 90% Confidence Limit of Mean

amount of free hydroxyproline present in the plasma of two groups of animals whose exposure to strontium began in utero and continued through the nursing period and in one group) that is continuing on daily feeding of 125 μ Ci. Those animals who received intrauterine exposure only were lower than the controls but not as depressed as the others. The animals who began receiving ^{90}Sr through their foster dams' milk while nursing and then were placed on a daily regimen of 125 μ Ci ^{90}Sr were also slightly lower than the control animals.

CONCLUSIONS

Offspring from dams ingesting 125 μ Ci daily evidenced a reduction

in free hydroxyproline when their exposure to ^{90}Sr began at conception and continued into adulthood via daily ingestion of 125 μ Ci or when their ingestion of ^{90}Sr ceased at weaning. There were no differences in protein-bound or peptide-bound hydroxyproline.

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ALKALINE PHOSPHATASE ISOENZYMES IN ^{90}Sr -TREATED SWINE

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Studies on serum alkaline phosphatase isoenzymes were initiated utilizing a differential inhibition method. Preliminary results indicate that the intestinal isoenzyme occurs most frequently in swine serum. Three-year-old swine receiving ^{90}Sr exposure only during nursing exhibited higher serum activity, presumably due to the intestinal isoenzyme, than did animals receiving uterine, milk, and 125 μ Ci/day ^{90}Sr exposure.

Partition values for alkaline phosphatases in serum are useful in determining the origin and fate of the

serum enzymes in acute and malignant diseases. The level of serum activity in transient elevations is controlled

by the rate of release from damaged tissue and the rate of clearance from the circulation. In man, increased alkaline phosphatase levels are observed in childhood, pregnancy, liver diseases, and in bone disorders associated with osteogenic processes. An unusual isoenzyme, the **Regan** enzyme, **has been** detected in cancer patients.(1)

Although elevated serum alkaline phosphatase levels occur sporadically in most animal populations, the appearance and persistence of increased levels of enzyme activity in a group of swine, whose only ^{90}Sr -exposure was acquired by nursing from sows fed $125\text{ }\mu\text{Ci }^{90}\text{Sr/day}$, seemed somewhat unusual. Figure 1 compares the average enzyme values from these animals

with groups receiving the following ^{90}Sr exposures: uterine; uterine and milk; milk and $125\text{ }\mu\text{Ci }^{90}\text{Sr/day}$ feeding regimen; and uterine, milk, and ^{90}Sr feeding regimen.

A study was initiated to determine the origin of the major isoenzyme in **the** serum alkaline phosphatase pool and to obtain preliminary data to assess the prognostic value of isoenzymes in studying the effects of ^{90}Sr on bone, liver, and intestine.

OBSERVATIONS

Serum alkaline phosphatase isoenzymes were identified by means of differential inhibition, utilizing urea and L-phenylalanine (LPA). (1) In man, the order of resistance to

FIGURE 1. Serum Alkaline Phosphatase Values in Offspring of Dams Fed $125\text{ }\mu\text{Ci }^{90}\text{Sr/Day}$ and Control Animals

inhibition by urea is: placental = intestinal > liver > kidney > bone.⁽²⁾ Human intestinal and placental alkaline phosphatases are very susceptible to LPA inhibition.⁽³⁾ To determine if the same degree of inhibition was demonstrated by porcine tissue, a few necropsy specimens were obtained and analyzed for alkaline phosphatase in the absence and presence of these inhibitors. Results are shown in Table 1. From these few values, it is evident that there may be some variance in inhibition between human and porcine phosphatases under these particular conditions and that additional porcine tissue values should be established. In swine the order of resistance to urea inhibition appears to be intestinal > liver = kidney = bone, so that the latter three isoenzymes may not be distinguishable with urea. LPA inhibits porcine intestinal alkaline phosphatase to the greatest degree and kidney enzyme the least.

Ranges of values (upper or lower 95% confidence limits) for porcine tissue alkaline phosphatase inhibition were utilized in constructing

arbitrary limits for isoenzyme indicators in serum. Ranges of values falling between limits were considered to be mixtures of isoenzymes. These values, as well as the frequency at which they occurred in a total of 34 serum samples from separate animals, are shown in Table 2. Since the appearance of the kidney enzyme in serum is rare, at least in man, this isoenzyme may not be a contributor to the swine serum pool. No pregnant animals were included in the study so the effect of inhibitors on placental enzyme was not tested.

Examination of the observed frequency of the serum samples classified according to arbitrary ranges shows a large discrepancy between the two inhibitors. This could, no doubt, be corrected by determining tissue and serum values in a larger population of control animals of various ages. Nevertheless, the values demonstrate a preponderance of intestinal isoenzyme in the serum pool.

A comparison of serum alkaline phosphatase values with three treatment groups of the ⁹⁰Sr-exposed swine is shown in Figure 2. The isoenzyme

TABLE 1. Alkaline Phosphatase Values in Porcine Tissue

Tissue	Number of Samples	Activity ^(a) per mg N	Percent Original Activity	
			Urea	L-Phenylalanine
Bone	1	6.0	13	76
Liver	3	76 ± 9	11 ± 1.7	69 ± 0.9
Kidney	3	249 ± 51	16 ± 3.2	91 ± 4.8
Intestine	3	252 ± 53	51 ± 7.6	32 ± 2.3

a. Bessey-Lowry-Brock units

TABLE 2. Arbitrary Range and Observed Frequency of Alkaline Phosphatase Isoenzymes in Porcine Serum

Isoenzyme Indication	Percent Original Activity		Percent Observed Frequency	
	Urea	L-Phenylalanine	Urea	L-Phenylalanine
Bone, liver, or kidney	0-24	67-100	9	3
Mixture	25-31	39-66	0	38
Intestine	32-100	0-38	91	59

FIGURE 2. Comparison of Serum Alkaline Phosphatase Values with Isoenzyme Indication in ⁹⁰Sr-Treated Swine at 36 Months of Age

indication does not necessarily correlate with the serum activity, but serum levels greater than ten Bessey-Lowry-Brock units apparently can be attributed to the intestinal isoenzyme. A comparison between treatments shows that the group receiving ^{90}Sr in milk (from birth to weaning) may have a larger number of animals displaying intestinal isoenzyme in the serum than the group that received uterine, milk, and $125\text{ }\mu\text{Ci/day }^{90}\text{Sr}$ exposure. Serum activity in more individuals of the latter treatment could be attributed to a mixture of isoenzymes. Terminal serum samples from three animals that had been anorectic for several days also indicated that the phosphatase activity was due to a mixture of isoenzymes.

The elevated serum alkaline phosphatase values which can be attributed to the LPA-sensitive isoenzyme may possibly be attributed to the hypersecretion of gastrointestinal fluids or the reabsorption of intestinal alkaline phosphatase either directly or via the lymph.⁽¹⁾ Conversely, ^{90}Sr -exposure may inhibit secretion or reabsorption to produce lowered intestinal isoenzyme values. Since this inhibitor method cannot distinguish between the Regan, placental, and intestinal isoenzymes, there is also a possibility that tumors may be developing in some of these animals.

CONCLUSIONS

Additional studies on swine tissue and serum alkaline phosphatase activ-

ity should be performed to establish limits for isoenzyme indicators in serum.

Results from the current studies, using arbitrary limits, show that the intestinal isoenzyme is probably the predominant isoenzyme in swine. Daily ^{90}Sr feeding may tend to lower the contribution of this enzyme to the serum pool so that these studies may be of significance in the development of intestinal tumors, as suggested in swine fed $25\text{ }\mu\text{Ci }^{90}\text{Sr/day}$. Emphasis should also be placed on the identification of a swine isoenzyme comparable to the Regan isoenzyme in man. This enzyme is found in human patients with cancer and metastases and has an inhibitor pattern similar to placental isoenzyme. Effects of other enzyme inhibitors, such as heat or possibly electrophoretic separation utilizing cellulose acetate or polyacrylamide gel, should be used to clarify placental-intestinal and liver-bone isoenzyme distributions in swine.

Isoenzyme studies in animals from the perinatal period to weaning should be particularly sensitive indicators of organ damage since the phosphatase activity is changing so rapidly.

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EFFECTS OF ^{90}Sr ON PHYTOHEMAGGLUTININ STIMULATION
OF PERIPHERAL LYMPHOCYTES OF MINIATURE SWINE

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The in vitro response of Zeukocytes to the mitogen phytohemagglutinin was studied in swine receiving ^{90}Sr during various intervals of their life-span. No significant differences among treatments were observed, but individual animals receiving ^{90}Sr exposure in utero alone or in combination with milk exposure and those on the ^{90}Sr feeding regimen had a tendency to exhibit lower values for response indices.

The in vitro response of peripheral leukocytes to the mitogen phytohemagglutinin (PHA) has been employed as a measure of cellular immunocompetence. Previous studies⁽¹⁾ have demonstrated that the leukocytes of swine fed 625 μCi ^{90}Sr /day fail to respond to this mitogen 2-3 months after initiation of the feeding regimen. Since animals exposed to a ^{90}Sr -level of 125 μCi /day have a higher probability of developing hematopoietic neoplasms, the in vitro response of peripheral leukocytes to PHA stimulation was measured in swine ingesting 125 μCi daily and in offspring who were exposed in utero and/or from milk of dams who were ingesting ^{90}Sr .

OBSERVATIONS

Heparinized blood samples were obtained from 3-year-old swine receiving the following treatments: in utero

exposure in a dam fed 125 μCi ^{90}Sr /day (U) ; milk from a 125- μCi /day dam (M) ; in utero and milk (U+M); milk from suckling and a 125- μCi /day feeding regimen continuously from weaning (M+F); or in utero, milk, and the feeding regimen (U+M+F). The erythrocytes were allowed to settle and 1 ml of leukocyte-enriched plasma was inoculated into culture medium consisting of 5 ml of NCTC 109 containing penicillin-streptomycin and fetal calf serum. Medium was prepared with and without PHA. Incubation was carried out at 37 °C for 64 hr and colchicine was added to the cultures, which were then incubated for an additional period of 4 hr. At harvest, the cells were treated with 0.85% sodium citrate solution, fixed in a mixture of 3:1 methanol-acetic acid, dropped on slides and stained with Giemsa. Leukocyte concentrations were determined in peripheral blood with a Coulter

Model S and in the plasma inoculum and the culture medium by means of a Coulter Model B. Mitotic indices were obtained by observing the number of metaphases in 1000 cells.

Mean peripheral leukocyte and lymphocyte values for the treatment groups are shown in Figure 1a. Leukocyte values were significantly lower in animals on the daily ^{90}Sr feeding regimen (M+F and U+M+F) so that cellular concentrations in the culture inoculum and harvest were correspondingly lower than that of the other treatments (Table 1). However,

on a percentage basis, cellular values in unstimulated cultures (no **PHA**) were remarkably constant, ranging between 63 and 70% of the original number inoculated (Figure 1b). Values for mitogen-stimulated cultures were suggestive of a decreased response in leukocytes of animals receiving the longest exposure to ^{90}Sr (U+M+F) but were not significantly lower.

When the average ratios of total leukocytes in the **PHA** culture to those in the unstimulated culture are compared (Table 1), no significant differences are observed; but the control

FIGURE 1. A. Mean Leukocyte and Lymphocyte Values in Peripheral Blood B. Mean Values for the Percentage of Inoculated Cells in Unstimulated and PHA-Stimulated Cultures

TABLE 1. Leukocyte Values in the Inoculum and in Stimulated and Unstimulated Cultures

Parameter	⁹⁰ Sr-Exposure					
	None	Uterine	Milk	Uterine + Milk	Milk + ⁹⁰ Sr Feeding	Uterine + Milk + ⁹⁰ Sr Feeding
Number of Animals	3	9	10	8	4	7
Leukocytes in Inoculum (10 ⁶ /ml)	16.1 ± 1.76 ^(a)	18.5 ± 3.01	17.7 ± 1.70	15.8 ± 2.92	10.1 ± 1.64	8.9 ± 0.71
Total Leukocytes with PHA (10 ⁶ /ml)	16.9 ± 2.38	16.7 ± 2.09	17.6 ± 1.14	13.7 ± 1.54	8.9 ± 1.27	7.1 ± 0.72
Total Leukocytes without PHA (10 ⁶ /ml)	9.9 ± 0.58	12.8 ± 2.59	11.6 ± 1.12	10.9 ± 0.61	6.9 ± 1.58	6.1 ± 0.53
Leukocytes with PHA/Leukocytes without PHA (X 100)	171 ± 25	149 ± 14	157 ± 8.0	126 ± 12	135 ± 12	123 ± 19
Percentage of Metaphases in PHA Culture	3.2 ± 0.80	1.6 ± 0.34	2.3 ± 0.25	2.1 ± 0.49	2.5 ± 0.25	2.2 ± 0.19

a. . Mean ± standard error of the mean.

group had the highest ratio and the U+M+F group had the lowest. The distribution of these values for individuals within treatments (Figure 2A) shows that values less than 100, indicating a loss of cells in the PHA culture rather than an increase, are observed only in those animals subjected to uterine ⁹⁰Sr exposure (U, U+M, U+M+F). However, the swine in the M+F group were the four survivors of ten original animals. Consequently, the results might have been quite different if this study had been performed at an earlier time period.

Mean values for percentage of metaphases in the PHA-stimulated culture are shown in Table 1. No significant differences among treatments were observed. Slides from cultures contain-

ing no PHA were scored for 16 of the 41 animals. A maximum of 0.1% metaphases was obtained for six animals, while no metaphases were observed in ten preparations.

The distribution of individual values for the total number of metaphases in the PHA culture reflects both the slight depression in percentage of metaphases and the decrease in total cells in culture so that lower values are observed in animals receiving the highest ⁹⁰Sr exposure. Less than 100,000 metaphases were obtained in individuals in each of the three groups receiving uterine exposure. No direct correlation between percentage of metaphases and total cell numbers in the PHA culture could be made for individual animals.

FIGURE 2. Distribution of Individual Values A. Ratio of Leukocytes in Stimulated and Unstimulated Cultures B. Total Number of Metaphases in PHA Cultures Cultures

CONCLUSIONS

Although no statistically significant differences between treatments were observed in this study, indices involving the number of leukocytes and metaphases in PHA cultures had a tendency to be lower in animals receiving the longest exposure to ^{90}Sr or in those receiving uterine exposure. Since this study was performed with 3-year-old swine, six of ten in the milk and ^{90}Sr feeding and two of nine in the uterine, milk, and feeding group had succumbed to the treatment. Serial observations on individual animals and the accumulation of larger numbers of control indices would be helpful in assessing treatment differences.

McFee and Banner⁽²⁾ found a correlation between percentage of tritium-labelled cells and metaphases in PHA-stimulated cultures of swine leukocytes so that labelling studies, performed concurrently with the determination of numbers of mitotic cells or even nuclear volumes, might provide more information on cell populations in these animals.

Recent studies by Abdou and Abdou⁽³⁾ have shown that PHA stimulates the thymus-dependent immunocompetent cells but not bone marrow cells, and the response to pokeweed mitogen and anti-serum to immunoglobulin may be an index for the bursal dependent system in man. Studies to investigate marrow-derived cells should be performed in swine, especially in those animals

receiving bone-seeking emitters. If the ultimate effect is caused by immunosuppression, we should be able to measure this if we look at the correct cell population or parameter.

The highest percentages of mitotic cells and labelled cells were observed after an 84-hr incubation period of stimulated swine leukocytes by McFee and Banner⁽²⁾. Since this study was performed on 68-hr cultures, maximum differences among treatments may not have been attained. Furthermore, the duration of a mitotic delay, if any,

as a function of the radiation dose should be explored.

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PORCINE AND HUMAN CHROMOSOME ABERRATIONS INDUCED BY IN VITRO EXPOSURE TO X-RAYS OR ⁹⁰Sr-⁹⁰Y

Investigator:

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The coefficients of aberration determined for two-break abnormalities produced by a single exposure to X rays prior to stimulation by phytohemagglutinin suggest that swine chromosomes are slightly more resistant to injury than human chromosomes. Lymphocytes cultured in the presence of ⁹⁰Sr-⁹⁰Y showed a dose dependent production of both chromatid and isochromatid aberrations.

One of the criteria for assessing radiation-induced damage to an individual is the production of chromosomal aberrations. The preliminary data presented here were obtained from experiments comparing the response of porcine and human leukocyte cell cultures to in vitro radiation from X rays and ⁹⁰Sr-⁹⁰Y. These com-

parative studies will aid in extending the effects of chronic ⁹⁰Sr-⁹⁰Y ingestion observed in swine to the evaluation of hazards of deposited radionuclides in humans.

OBSERVATIONS

Samples of plasma containing leukocytes, prepared from freshly drawn

whole blood obtained from female Pitman-Moore swine and a human female volunteer, were irradiated with 50, 100, or 200 R X-ray. Following irradiation, the specimens were cultured according to the method of Moorhead, et al.⁽¹⁾ After 45 hr incubation at 37 °C, colchicine was added to arrest mitosis in metaphase and 4 hr later the cells were harvested. The slides were blaze dried and stained with 2% Giemsa in phosphate buffer, pH 6.8. Suitable metaphases were photographed under oil immersion.

Strontium-90 irradiation was accomplished by adding carrier-free ^{90}Sr - ^{90}Y in 0.01 N HCl to a solution of NCTC 109. Appropriate amounts of this stock solution were added to

cells suspended in the complete culture medium such that the cell cultures contained 7.4 $\mu\text{Ci/ml}$, 15 $\mu\text{Ci/ml}$, or 29 $\mu\text{Ci } ^{90}\text{Sr}$ - $^{90}\text{Y/ml}$ in a total volume of 7 ml. Incubation, cell harvest, and slide preparation were the same as described for the X-irradiation.

The data in Table 1 summarize the preliminary results from three in vitro irradiations. As expected, the incidence of chromatid aberrations in both porcine and human cells was not significantly increased by X-irradiation when the cells were irradiated prior to stimulation by phytohemagglutinin. This is consistent with the observation that circulating

TABLE 1. Chromosomal Aberrations Induced by In Vitro Irradiation

	No. of Cells Scored		CTD + CTG ^(a)		CD + AL ^(a)		Rings and Dicentrics	
	P ^(b)	H ^(b)						
			P	H	P	H	P	H
X Ray								
Control	160	94	3	4	0	0	0	0
50 R	168	139	2	5	4	6	2	2
100 R	171	186	2	10	5	10	4	8
200 R	165	171	5	7	17	26	6	25
⁹⁰ Sr- ⁹⁰ Y								
Control	133		3		1		0	
7.4 μCi/ml	152		6		5		2	
15 μCi/ml	169		40		11		1(1) ^(c)	

a. CTD, chromatid deletions. CTG, chromatid gaps. CD, chromosome deletions. AL, acromatid lesions on both arms.

b. P, porcine. H, human.

c. Number in parenthesis refers to a quadricentric chromosome.

lymphocytes from normal, healthy individuals are in the resting or **pre-DNA** synthesis period, G_1 . Therefore, the chromatid aberrations scored here represent those occurring spontaneously, not as a consequence of irradiation. Pooling the chromatid aberrations for the various exposures yielded spontaneous aberration rates of 0.018 **aberrations/cell** for swine and 0.044 **aberrations/cell** for humans. The production of chromosomal aberrations was dose dependent. The average one-break aberration coefficients calculated from these data were $0.43 \times 10^{-3} \text{ cell}^{-1} \text{ R}^{-1}$ for pig and $0.72 \times 10^{-3} \text{ cell}^{-1} \text{ R}^{-1}$ for human cells. For two-break aberrations, the coefficients obtained were $0.27 \times 10^{-5} \text{ cell}^{-1} \text{ R}^{-2}$ for pig and $0.46 \times 10^{-5} \text{ cell}^{-1} \text{ R}^{-2}$ for human.

The production of both chromatid and isochromatid aberrations was dose dependent when the cells were irradiated throughout their cell cycle by ^{90}Sr - ^{90}Y , in contrast to the effect seen when the cells were X-irradiated in the G_1 stage. The highest dose, 29 $\mu\text{Ci/ml}$, caused extensive cell death so that an insufficient number of metaphases were examined and no data are available at this time. At the lower dose of 15 $\mu\text{Ci/ml}$ there were significantly more chromatid aberrations than chromosomal abnormalities, while at the lower dose of 7.4 $\mu\text{Ci/ml}$ the numbers of chromatid and chromosome abnormalities were approximately equal. Data from irradiation of human cells are not available for presentation at this time.

CONCLUSIONS

The preliminary data presented here on chromosome aberration yields produced by X-irradiation suggest that swine chromosomes are slightly less susceptible to radiation damage than human chromosomes. The one-break aberration coefficients of $0.43 \times 10^{-3} \text{ cell}^{-1} \text{ R}^{-1}$ for swine and $0.72 \times 10^{-3} \text{ cell}^{-1} \text{ R}^{-1}$ for human are in general agreement with those reported for swine of $1.08 \times 10^{-3} \text{ cell}^{-1} \text{ R}^{-1}$ by McFee, et al.⁽²⁾ and $2.4 \times 10^{-3} \text{ cell}^{-1} \text{ R}^{-1}$ by Bender and Gooch⁽³⁾ for human. McFee, et al. reported a lower two-break coefficient of $0.09 \times 10^{-5} \text{ cell}^{-1} \text{ R}^{-2}$ compared to $0.27 \times 10^{-5} \text{ cell}^{-1} \text{ R}^{-2}$ reported here for pigs. Bender and Gooch calculated a value of $0.52 \times 10^{-5} \text{ cell}^{-1} \text{ R}^{-2}$ for human chromosomes which is in good agreement with the value of $0.46 \times 10^{-5} \text{ cell}^{-1} \text{ R}^{-2}$ obtained in this study. Further studies using both ^{90}Sr and X rays are expected to reinforce these observations on the relative susceptibility of swine and human chromosomes to radiation.

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SEQUESTRATION OF HEAT-DAMAGED ERYTHROCYTES AS A MEASURE
OF RETICULOENDOTHELIAL FUNCTION IN CATS. INGESTING ^{90}Sr DAILY

Investigator:

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Technical Assistance:

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The ability of the reticuloendothelial system to phagocytize damaged erythrocytes did not appear to be impaired in cats chronically ingesting ^{90}Sr and, in fact, may have been hyperphagocytic. The sequestration of heterologous cells by pulmonary cells was equal to that by cells of the liver and spleen.

There are conflicting reports regarding the reticuloendothelial system (RES) and its ability to phagocytize colloidal particles after X-irradiation. In some studies, it appears increased; in others, decreased; and in still others, is apparently unaffected by external irradiation.

This study was undertaken to determine if chronic ^{90}Sr feeding altered the RES of cats. A functional change would be indicated by an increased or decreased ability to sequester a cohort of heat-damaged ^{51}Cr -labeled erythrocytes. The reinfusion of injured cells is, in many respects, a more physiologic assay of RES function than infusion of colloidal particles. Also, the small volume of injured cells does not saturate the phagocytic function of the RES; and the isotopic label facilitates convenient and accurate quantitation.

Mature cats were fed 0.2 μCi ^{90}Sr daily for 6 months. They were then

randomly assigned to three treatment groups to receive heat-damaged ^{51}Cr -tagged erythrocytes as follows: homologous, heterologous (porcine), and sensitized to porcine erythrocytes 1 month prior to heterologous cells. Use of homologous cells should test the ability of the RES to phagocytize slightly damaged erythrocytes, simulating the physiologic sequestration of senescent erythrocytes; whereas, the heterologous cells in non-sensitized and sensitized cats should test the ability to phagocytize foreign protein and the immunologic reaction, respectively. The homologous and heterologous blood was collected aseptically in acid-citrate-dextrose anticoagulant and centrifuged for removal of plasma and buffy coat. The red cells were then incubated with ^{51}Cr for 30 min, ascorbic acid was added, and then the cells washed two times in Earle's solution to remove unbound ^{51}Cr . The red cells were resuspended to a volume of packed red

cells (VPRC) of approximately 50 and then incubated at 50 °C for exactly 20 min prior to re-infusion via an indwelling jugular cannula.

Blood and plasma samples were obtained 15 min after injection, and again at 120 min, at which time the animals were killed to determine tissue distribution of the ^{51}Cr -tagged cells. In order to preclude the effects of uneven isotope distribution, each organ was minced and an aliquot

taken for ^{51}Cr assay. Radioanalysis of the extravascular tissue at the injection site was made and the data from certain animals eliminated if ^{51}Cr leakage was evident.

OBSERVATIONS

Blood and plasma concentrations are illustrated in Figure 1; tissue concentrations in Figure 2. Although the

FIGURE 1. Red Blood Cell and Plasma Concentrations of ^{51}Cr Following Injection of Homologous or Heterologous Heat-Damaged ^{51}Cr -Tagged Erythrocytes into Control Cats and Those Exposed to ^{90}Sr by Daily Ingestion (Individual and Mean Values)

FIGURE 2. Organ Sequestration 2 Hr After Injection of Homologous or Heterologous Heat-Damaged ^{51}Cr -Tagged Erythrocytes into Control Cats or Those Exposed to ^{90}Sr by Daily Ingestion (Individual and Mean Values)

number of animals per group was small and individual variation in some tissues quite large, it appears there are few differences between ^{90}Sr and control cats. However, the clearance of tagged, heterologous cells in the ^{90}Sr cats was faster at the 15- and 120-min periods in both sensitized and unsensitized animals than in their controls and much faster in all four of these

groups than in those given homologous cells. The processing of these damaged cells by the RES was also apparently increased as evidenced by the plasma concentrations at corresponding periods. There are, however, no differences in plasma concentrations between control and ^{90}Sr cats although the concentration is higher in the sensitized animals.

Since the liver and spleen concentrations are lower in the sensitized cats, and the kidney much greater, this may indicate a different process is involved and that the renal clearance becomes more important in eliminating the end product.

The surprising tissue concentration of labeled cells was that found in the lung. The concentration of heterologous cells was approximately ten times that of the homologous cells; but, even in the latter group, of animals, it was much higher than bone marrow.

CONCLUSIONS

Chronic ingestion of ^{90}Sr by cats had no inhibitory effect on phagocytosis of heat-damaged erythrocytes. In fact, evidence with heterologous cells indicated there was more rapid clearance from their circulating

blood than in the control animals. Also, with infusion of homologous cells, the liver and spleen concentrations were approximately two times greater in the ^{90}Sr fed cats than in their controls. Again this would indicate an increased phagocytic function of the RES in these organs.

The lung sequestration of heterologous cells (and even homologous cells) is of particular interest. Although not of special interest in these animals, it would appear that this could be utilized as an assay of the pulmonary RES function during inhalation-effects studies. Using an isotopic label, the pulmonary concentration could be monitored at intervals with scanning equipment. At least, under the conditions of this experiment, it appears the phagocytic function of the lung in regard to foreign cells is equal to that of the liver or spleen.

RADIATION-INDUCED LEUKEMOGENESIS

The hematopoietic disorders observed in the miniature swine of our chronic ^{90}Sr toxicity experiment have ranged from myeloid metaplasia to granulocytic leukemia to lymphocytic leukemia, with hematologic variables in each group. These cases represent the largest number of experimentally induced hematopoietic disorders ever produced in large experimental animals and afford an excellent model system for basic studies, not only on radiation-induced leukemia, but also on the myeloproliferative and lymphoproliferative disorders of man and leukemogenesis in general.

The objectives of this project are to further characterize these leukemias by determining if they have a viral etiology. This objective is being approached by isolating viruses from pigs with ^{90}Sr -induced leukemia, identifying these viruses, adapting them to tissue culture systems, and by eventually passing them to immunologically depressed, unirradiated animals in an attempt to transmit the leukemias. The study is also concerned with detection of immunological factors that might be responsible for the initiation and progression of these diseases.

RADIATION-INDUCED LEUKEMOGENESIS IN MINIATURE SWINEI. Isolation and Characterization of C Type Particles from Leukemic Swine

Investigators:

M. E. Frazier, J. R. Pratt, B. Rosario, and R. N. Ushijima*

The type C virus isolated from leukemic miniature swine that had been chronically exposed to ^{90}Sr was partially characterized. This virus was shown to have chemical, physical, and morphological properties similar to those of viruses known to cause leukemia in other animal species.

Virus particles have been observed in electron micrographs of plasma pellets and cell cultures from leukemic miniature swine chronically fed ^{90}Sr . The experiments described in this report were initiated to purify and characterize these C type virus particles. Our objective is to examine the possible role of such a virus in the induction of leukemia by radio-nuclides.

OBSERVATIONS

As reported previously (Annual Report, 1970), the porcine type C virus replicates in cell cultures and can be transmitted to other cell cultures. Supernatants of virus-infected porcine embryonic kidney cells served as the starting material from which the virus was extracted. The cells were removed by low speed centrifugation and the supernatants utilized for the experiments. A Spinco Ti-15 zonal

rotor was employed to concentrate the virus in sucrose gradients. The resulting virus-enriched preparation was further subjected to isopycnic banding in sucrose gradients.

Light-scattering patterns formed during isopycnic centrifugation were photographed. The density of the band was determined from its position relative to buoyant density markers. Gradients were examined by collecting fractions from the bottom of the tube. Aliquots of these samples were subsequently examined using UV spectrophotometry (Figure 1) and electron microscopy. The resulting virus preparations appeared to be fairly homogeneous (Figure 2) and, therefore, suitable for nucleic acid determinations.

The purified virus preparations were pelleted to remove any extracellular nucleic acid. Nucleic acid was then extracted by the phenol-SDS method and then precipitated from the aqueous solution with ethanol. The absorbance spectrum of the extracted nucleic acid (as shown in Figure 3) is consistent with the nucleic acid

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FIGURE 1. Optical Density of Gradient Fractions After Isopycnic Banding of Virus Particles Found in Cell Cultures from Leukemic Swine (This is a UV absorption profile of the porcine virus centrifuged through a preformed gradient of sucrose. The vertical arrows locate the position of the density markers, and the horizontal arrow indicates the fraction utilized for electron microscopy.)

FIGURE 3. Absorbance of the Porcine Virus RNA as a Function of Wave Length

FIGURE 2. Virus Particles from Leukemic Swine After Isopycnic Banding Following Continuous-Flow Centrifugation [Chemical analyses were performed on preparations following isopycnic banding experiments. Particles measure 90-110 nm in diameter. The "tails" displayed by some particles are probably due to osmotic factors (arrows). The line at the bottom of the picture represents 100 nm]

component of the porcine virus, being a single stranded RNA. The orcinol reaction was utilized to verify that the nucleic acid component was indeed RNA.

CONCLUSIONS

Chemical, morphological, and physical studies conducted on the virus particles found in plasma pellets and cell cultures from leukemic swine show them to be comparable to known leukemia agents. The density of these particles is approximately 1.12 gm/cm^3 . Their morphology is similar to known leukemia viruses, having an electron dense nucleoid and surrounding unit membrane.

The particles measured between 85 and 100 m μ in diameter. Chemical analyses suggest that these particles contain a single stranded ribonucleic acid core.

The leukemogenic and/or oncogenic potential of these virus particles in swine requires further studies.

RADIATION-INDUCED LEUKEMOGENESIS IN MINIATURE SWINE

II. Immunological Studies

Investigators:

*M. E. Frazier, J. R. Pratt,
and R. N. Ushijima**

Control and ^{90}Sr -exposed swine were sensitized to complete Freund's adjuvant, and cellular immunity was determined, using the macrophage migration inhibition technique. These studies indicate that chronic exposure to ^{90}Sr has an inhibitory effect on the normal cellular immune response of miniature swine.

It has been demonstrated that peripheral leukocytes, obtained from animals exhibiting delayed hypersensitivity, are inhibited from migrating in vitro by specific antigens. (1) The so-called Macrophage Migration Inhibition (MMI) test has been utilized as a measure of cellular immunity. Impaired immune responsiveness is often associated with malignant disease; therefore, we have undertaken a series of investigations to evaluate the effect of internally deposited radionuclides on normal immune mechanisms.

OBSERVATIONS

Cellular immunity was determined in swine immunized using Freund's adjuvant containing heat-killed mycobacteria. Three weeks after immunization, heparinized blood was collected and the leukocyte-rich plasma layer harvested for use in these studies. After washing the leukocytes three times, the cells were resuspended in medium to a concentration of 10^8 wbc/ml. Microcapillaries were filled with these cells and one end of the capillary plugged with wax. The tubes were centrifuged to lightly pack the cells. The capillary tubes were then cut at the cell-fluid interface, placed in

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growth chambers, and medium added with or without 30 μ g Protein Purified Derivative* (PPD) per ml. The chambers were incubated at 37 °C in an atmosphere of 95% air and 5% CO₂. After 18 hr, the cultures were removed, the area of migration was determined, and the migration index calculated.

The unimmunized control animals had a mean migration index (MI) of 97%, as compared to a 59% value in the immunized control group (Table 1). Thus, normal migration of peripheral leukocytes from control swine immunized with Freund's adjuvant was inhibited as expected. However, the values obtained from the immunized ⁹⁰Sr group gave an average MI of 85% as compared to 91% in the unimmunized ⁹⁰Sr animals. The meaning of the lower MI in the unimmunized ⁹⁰Sr group of animals is not clear, but the data from immunized ⁹⁰Sr swine indicate that cellular immunity is adversely affected by ingestion of 125 μ Ci/day of ⁹⁰Sr, as measured by the MMI test.

CONCLUSIONS

Preliminary results indicate that chronic exposure to internal doses of ⁹⁰Sr results in an inhibition of cellular immunity, as measured by the

* PPD is a heat stable protein extractable from mycobacteria species that retains specific antigen determinants of the protein found in the infecting bacilli.

TABLE 1. In Vitro Migration of Peripheral Leukocytes from Miniature Swine After Immunization with Complete Freund's Adjuvant

Animal No. and Description of Experimental Group	Migration Index =	
	Migration Area of Peripheral Leucocyte Cultures in Medium Containing 30 μ g/ml PPD	x 100
	Migration Area of Peripheral Leucocyte Cultures in Standard Medium	
Control - not immunized		
4018		87.3
4022		104.7
4152		102.2
4153		97.8
4154		95.0
	Mean	97.4
Control - immunized with Freund's complete adjuvant		
4095		49.2
3905		65.2
4020		76.1
4041		51.2
4151		51.7
	Mean	58.7
125 μ Ci/day 90 Sr - not immunized		
4004		92.2
4007		99.0
3987		82.3
3573		94.6
3583		87.7
	Mean	91.2
125 μ Ci/day 90 Sr - immunized with Freund's complete adjuvant		
4008		83.7
4012		81.6
4029		81.2
4032		87.3
4054		85.2
	Mean	84.0

macrophage migration test. Studies are underway to further explore these findings and determine the relationship, if any, between impaired immunity and the radiation-induced leukemia of miniature swine.

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INHALATION STUDIES

This project is concerned with determining the consequences of inhaling plutonium and other alpha emitters - the biological responses and the dose relationships. It is concerned with establishing parameters by means of which the hazards of human exposure may be recognized and evaluated. And, it is concerned with the development of effective therapy procedures for removing inhaled radionuclides from the body. A variety of investigations are included in this project, ranging from basic studies of lung biochemistry to life-time studies of retention and toxicity in beagle dogs.

Effort on this project was somewhat restricted due to emphasis on initiation of the "Low-Level Plutonium Inhalation Study in Beagles." No report is included on the long-term toxicity study, now in its thirteenth year, because no important changes have occurred.

PROBLEMS ASSOCIATED WITH THE CHARACTERIZATION OF $^{239}\text{PuO}_2$ AEROSOLS
GENERATED FOR INHALATION TOXICITY STUDIES WITH EXPERIMENTAL ANIMALS

Investigators:

*D. K. Craig and J. M. Thomas**

Technical Assistance:

W. G. Morrow and L. R. Richardson

The most important factor determining percentage inhaled $^{239}\text{PuO}_2$ deposited in the lungs of experimental animals is the particle size distribution of the aerosol. In one method of size characterization used, the projected diameter of particles on precipitator samples was measured. It was assumed that the $^{239}\text{PuO}_2$ was normally distributed with respect to the logarithm of particle size and estimates were made of the count median diameter (CMD) and the geometric standard deviation (GSD) of each aerosol. To investigate the reliability of this technique, analyses of the same set of electron micrographs were carried out with a Zeiss particle size analyzer by two different operators and with a Quantimet automatic electronic counter.

The results for both $^{239}\text{PuO}_2$ and a test aerosol comprising unaggregated spheres of Fe_2O_3 showed that the particle size distribution obtained was influenced both by the number of particles classified and by the magnification of the electron micrographs. Although the former had no significant effect on the calculated CMD of the aerosol, the chances of rejecting the hypothesis of log-normality increased as the number of particles classified increased. The GSD values calculated from the Quantimet data were significantly larger ($p < 0.05$) than those from the Zeiss data of either observer. The magnification of the electron micrographs affected the number of particles that could be seen per unit area of a sample of a PuO_2 aerosol.

Aerodynamic equivalent size classification of $^{239}\text{PuO}_2$ aerosols was carried out from cascade impactor samples. Results of a number of samples showed that there was a significant positive correlation ($p < 0.05$) between the $^{239}\text{PuO}_2$ concentration and the activity median aerodynamic diameter (AMAD) and a negative correlation between the AMAD and GSD values. The value obtained for the mass median diameter (MMD) for a particular $^{239}\text{PuO}_2$ aerosol from the impactor and from the projected diameter analyses differed widely.

INTRODUCTION

Studies involving the toxicology of inhaled Pu dioxide in beagle dogs have necessitated generation of $^{239}\text{PuO}_2$

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aerosols over a concentration range of several orders of magnitude. Since the time for which unanesthetized dogs can be subjected to nose-only inhalation exposures is limited to the approximate range 5 to 50 min, variation of the quantity of Pu inhaled by the dogs had to be achieved by changing the aerosol concentration.

The most important factor in determining the percentage of inhaled material that will be deposited in the alveolar regions of the lung is the aerodynamic size distribution of the aerosol.⁽¹⁾ Until recently, particle size characterization in our laboratory was carried out by classification of particles on samples suitable for optical or electron microscopic examination. It was considered necessary to investigate the reliability of these techniques when applied to non-spherical, frequently aggregated aerosols of unknown density such as $^{239}\text{PuO}_2$. We also wished to compare the results with those obtained from cascade impactor samples, which give the aerodynamic size distribution of the aerosol directly.⁽²⁾

METHODS

Aerosol generation was effected by nebulizing magnetically stirred suspensions of PuO_2 particles, the droplet cloud being dried in a heating column before being mixed with dilution air and passed into the inhalation exposure chamber. Samples for aerosol characterization were taken on membrane filters for concentration measurements by both thermal⁽³⁾ and point-to-plane electrostatic⁽⁴⁾ pre-

cipitation for diameter distribution analyses and by cascade impaction for aerodynamic size distribution determinations. Analyses of electron micrographs were carried out with both a Zeiss particle size analyzer and a Quantimet, the data being evaluated using a digital computer. On the Zeiss particle size analyzer, the diameter of a spot of light is adjusted by the operator until it matches the area of a particle on the micrograph. A lever is then depressed, which results in a hole being punched in the particle and one of 48 registers representing different particle diameters being activated. The Quantimet is an automatic image analyzing computer by means of which the particles on an electron micrograph are classified according to some preselected criteria, e.g., perimeter, width in a chosen direction, projected surface area, etc.

RESULTS

Projected Area Diameter

The particle size distributions computed on the Zeiss data from electron micrographs of three electrostatic precipitator (ESP) samples of $^{239}\text{PuO}_2$ are presented in Table 1. Five plates of randomly chosen areas were taken of each sample at each of two magnifications, 13,500 and 38,000, all particles on each set of plates being sized. For these analyses we assumed that the particles were normally distributed with respect to the logarithm of their diameters, i.e., log-normally distributed, and a χ^2 statistic was computed to test the validity of this assumption.

TABLE 1. Particle Size Distribution for Three $^{239}\text{PuO}_2$ Aerosols at Two Magnifications

Sample Type And #	Electron Micrograph Magnification = X 13500					Electron Micrograph Magnification = X 38000				
	Number Particles Sized	CMD ^(a) μm	GSD ^(b)	$\Sigma\chi^2$	.05 ^(d) Table Value	Number Particles Sized	CMD ^(a) μm	GSD ^(b)	$\Sigma\chi^2$	.05 Table Value
ESP #1 ^(c)	472	0.136	2.53	120.5	41.3	81	0.149	2.05	26.8	18.3
ESP #2	356	0.207	2.52	165.7	46.1	127	0.116	2.04	21.5	22.4
ESP #3	222	0.178	2.09	76.6	35.2	46	0.111	2.41	9.0	11.1

a. CMD = Count Median Diameter

b. GSD = Geometric Standard Deviation

c. Assuming log-normality, a CMD of 0.136 μm and a GSD of 2.53 gives a mass median diameter (MMD) of 1.8 μm .

d. Hypothesis of log-normality is rejected if $\Sigma\chi^2 > 0.05$ table value

The results indicated that acceptance of the hypothesis of log normality was directly proportional to the number of particles sized. Smaller counts resulted in a greater tendency for the hypothesis to be accepted than for instances where the number sized was large. We have repeatedly made this observation in sizing a number of different types of aerosols.

Some additional problems associated with characterization of particle size distributions for aerosols of this type are apparent from the composite of electron micrographs shown in Figure 1. The upper left plate shows a number of aggregates giving rise to differences in particle shapes on a thermal precipitator (TP) sample of a PuO_2 aerosol. From left to right along the top followed

by the bottom row, the following five plates focus on the same group of particles at magnifications in the ratio 1:2:3:4:5. Clearly, the higher magnifications show many small particles not visible at the lower two magnifications. The count median diameter obtained by sizing particles on plates like those would depend upon the magnification used.

Diameter Distribution of an Iron Oxide Aerosol

In order to investigate more carefully the analyses of particle size distributions from electron micrographs, we carried out a number of determinations on a sample of Fe_2O_3 , a fairly ideal aerosol comprising spherical particles (Figure 2) which are easy to size. Twenty electron micrographs (24,000X) of different

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FIGURE 1. Composite of Electron Micrographs of a TP Sample of a PuO_2 Aerosol (Upper left plate shows irregular shapes of particles. The remaining five plates show the same group of particles at magnifications in the ratio 1:2:3:4:5.)

areas of an ESP sample were classified with a Zeiss particle size analyzer and gave the cumulative distribution shown in Figure 3. The data were analyzed by two different statistical procedures; maximum likelihood method⁽⁵⁾ and log linear regression.⁽⁶⁾ Even though similar count median diameters* (CMD) were observed,

geometric standard deviations* (GSD) differed markedly. Moreover, the distribution of the iron oxide particles was not log-normal. The region between 20 and 80 cumulative percent illustrates the differences between predicted and observed frequency. Both procedures suffered from this deficiency but the maximum likelihood

* *Count Median Diameter = CMD = Diameter above and below which half of the number of particles lie = 50% size.*

* *Geometric Standard Deviation =*

$$\text{GSD} = \frac{84.13\% \text{ Size}}{50\% \text{ Size}} = \frac{50\% \text{ Size}}{15.87\% \text{ Size}}$$

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24,000X

FIGURE 2. Electron Micrograph of an ESP Sample of an Iron Oxide Sample, Shadowed with Chromium at 26°

method gave the better fit to observed data points. The number of particles classified, 1215, was large enough to ensure that a representative distribution was observed that was descriptive of the actual distribution of sizes for this iron oxide aerosol.

Particle size classification on the above 20 electron micrographs was carried out on the Zeiss particle size analyzer by two different observers and, also, on the Quantimet. The data for each plate were evalu-

ated by the maximum likelihood method, the means and standard deviations of the various CMD and GSD values being computed. The results are summarized in Figure 4. There were significant differences in the number of particles enumerated by the Quantimet and by the two observers using the Zeiss particle size analyzer, probably because the contrast level on the Quantimet must be arbitrarily set by the operator to ignore all features of lower contrast. The large

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FIGURE 3. Log-probability Plot of Zeiss Sizing Data of an Fe_2O_3 Sample, Showing Straight Lines Fitted by Two Different Statistical Techniques

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FIGURE 4. Summary of Results of Statistical Analysis of Size Distribution Determinations of an Iron Oxide Aerosol and a Plutonium Dioxide Aerosol

GSD values from the Quantimet were probably due to particles being placed in only a few different size categories, typically from four to six. However, the Quantimet is now capable of placing particles into 22 different size categories. We have not yet used it in this new operating mode.

In order to clarify the effect of the number of particles sized on the statistical analyses of the distribution (Table 1), we constructed four different samples from the 20 individual plates. In Table 2, results are presented for one randomly chosen plate, the pooled results of four randomly chosen plates of the remaining 19, for the remaining 15 plates, and for all 20 plates combined. Appropriate total numbers of particles sized were 83, 258, 874, and 1215, respectively. No significant differences in either CMD or GSD values were found in any of these four artificial samples. However, the Chi square goodness-of-fit test

shows that we would have accepted the hypothesis that the particle sizes were log-normally distributed for the smallest sample but would have rejected that hypothesis for the other three. The computed statistic becomes successively greater than the 0.05 table value as the number of particles classified increased. A second statistical test, the Kolomogorov-Smirnov (K-S) goodness-of-fit test, does not reject the hypothesis of log-normality in any of the above cases ($p < 0.05$). It is our experience, contrary to that of Ong and LeClare,⁽⁷⁾ that the K-S test is a relatively weak one because it rarely rejects any reasonable postulated distribution. However, it is advantageous because it is a nonparametric test.

Diameter Distribution of a $^{239}\text{PuO}_2$ Aerosol

We repeated the above procedure for a Pu dioxide sample. The numbers of particles classified by the two

TABLE 2. Effect of Sample Size on Statistical Analysis of an Fe_2O_3 Aerosol (30,000X)

Electron Micrograph Numbers Counted	Number Particles Sized in Sample	Maximum Likelihood Analysis		Goodness of Fit Analysis ^(a)		
		CMD	GSD	χ^2	.05 Table Value	Log-Normality Hypothesis Accepted
1	83	0.114	1.85	7.7	18.3	Yes
4	258	0.102	1.81	75.9	37.7	No
15	874	0.111	1.89	84.7	49.8	No
20	1215	0.109	1.87	125.1	49.8	No

a. The Kolmogorov-Smirnov goodness-of-fit test does not reject the hypothesis of log-normality in any of the above cases.

observers using the Zeiss particle size analyzer were 776 and 904, respectively, while the Quantimet classified 442 particles. The mean and standard deviations of the three sets of CMD and GSD values were computed as before and the results are also given in Figure 4. Once again, as for the Fe_2O_3 , the GSD's as determined by the Quantimet were significantly higher than those obtained on the Zeiss particle size analyzer.

However, whereas the CMD estimates obtained from the observer B and the Quantimet data were nearly the same, those obtained from the observer A data were somewhat larger. This is probably due to differences in judgment in matching the circular spot of light in the Zeiss particle size analyzer to particles of irregular

shape by different observers. This illustrates the uncertainty of the method. Linear correlation analyses conducted on the various sets of data (e.g., CMD values for individual plates from the data of A, B, and Q) gave no correlation coefficients that were significant at the 5% level for either the Fe_2O_3 or the PuO_2 aerosol, i.e., the variation of CMD and GSD from plate to plate was random.

Aerodynamic Size Distribution of PuO_2

Figure 5 is an electron micrograph of an ESP sample of a $^{239}\text{PuO}_2$ aerosol in which the Pu was obviously accompanied by some extraneous material, probably water from the nebulizer which had not yet evaporated. Some Pu particles appear to be dry whereas others appear to have been

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FIGURE 5. Electron Micrograph of an ESP Sample of a $^{239}\text{PuO}_2$ Aerosol, Shadowed with Chromium at 26°

TABLE 3. Aerodynamic Equivalent Size Distribution of $^{239}\text{PuO}_2$ Aerosols

	Aerosol Concentration (nCi/l)	Size Distribution from Impactor Samples	
		AMAD (μm)	GSD
1	0.54	1.32	1.87
2	0.59	2.42	2.17
3	1.14	1.58	1.88
4	1.84	2.60	1.99
5	4.22	1.56	2.12
6	5.25	1.78	2.40
7	5.56	1.28	1.92
8	7.01	2.06	1.77
9	7.04	2.22	1.77
10	7.33	1.79	2.11
11	8.66	1.68	1.82
12	11.8	1.60	1.72
13	13.3	2.22	1.87
14	18.3	2.39	2.11
15	19.3	2.01	1.96
16	20.1	2.67	1.86
17	20.1	3.01	1.90
18	27.7	2.00	1.95
19	29.0	2.50	1.81
20	36.6	2.73	1.77
21	42.2	2.44	1.77
22	43.5	2.19	1.81
23	53.2	2.06	1.79
24	60.1	2.14	1.85
25	61.9	2.30	1.83
26	62.5	2.60	1.86
27	66.0	2.77	1.59
28	67.1	2.07	1.74
29	93.5	2.98	1.54
30	137	2.64	1.84
31	165	2.72	1.86
32	171	2.68	1.58
33	187	2.12	1.87
34	301	1.94	1.80
35	314	2.78	1.76
36	357	2.75	1.73

associated with droplets. A determination by the optical techniques described above of the particle size distribution of such an aerosol is clearly very difficult. The chance that an inhaled particle will be deposited at a particular site depends not only upon the diameter of the particle or droplet, but also upon its effective density. For this reason and because of the extreme tedium in carrying out Zeiss particle size analyses on electron micrographs and the uncertainty of the results obtained, an aerodynamic method of determining particle size distributions of aerosols using the Mercer cascade impactor has recently been adopted. This device has seven successively smaller round hole jets and impaction stages and a backup membrane filter paper.

Samples are taken by drawing air through the impactor at a known rate. Under these conditions, the effective cutoff diameter for unit density spheres can easily be calculated for each stage.⁽⁸⁾ Radioactivity collected on the successive stages is determined and the results are evaluated by a computer program which uses the maximum likelihood method for calculation of activity median aerodynamic diameter (AMAD) and GSD. Some measured values and results for AMAD and GSD are given in Table 3. A significant positive correlation ($p < 0.05$) was observed between the aerosol concentration and AMAD. We also found a significant negative regression between the AMAD and GSD, the former decreasing as the latter increased. Both slope and intercept

of the equation (Figure 6) are significantly different from zero ($p < 0.05$). In other words, as the aerosol concentration increased, its aerodynamic median diameter increased, but the range of particle sizes decreased. A large number of further samples⁽¹⁾ have confirmed the validity of this observation, the reasons for which have been explored elsewhere in this Annual Report. We have also found that repeated impactor samples of the same aerosol give the same AMAD and GSD values to within a few percent.

A cascade impactor sample of the same $^{239}\text{PuO}_2$ aerosol from which the ESP Sample No. 1 of Table 1 was taken gave an AMAD = $2.07 \mu\text{m}$ and a GSD =

2.16. Assuming an effective density of 10.6 g/cm^3 for the PuO_2 particles, these values give an MMD = $0.56 \mu\text{m}$ and a CMD = $0.095 \mu\text{m}$ for a log-normally distributed aerosol. This CMD agrees quite well with that obtained from the ESP sample ($0.136 \mu\text{m}$), but the calculated MMD's ($1.8 \mu\text{m}$ for the ESP sample) compare poorly.

CONCLUSIONS

The number of particles sized in attempting to determine the particle size distribution of an aerosol has an important influence on the conclusions which one draws as to the definition of the distribution, although

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FIGURE 6. Graph of AMAD Versus GSD of $^{239}\text{PuO}_2$ Aerosols with Fitted Regression Line and 95% Confidence Interval of Line

it does not seem to have a significant effect upon the determination of count median diameter.

As the number of particles sized increased, the chances of rejecting a hypothesis of log-normality for the distribution of projected particle diameter increases. The χ^2 test is much more powerful than the Kolmogorov-Smirnov goodness-of-fit test, insofar as the latter is unlikely to reject the hypothesis of log-normality even though there may be wide discrepancies in observed and predicted values from such distributions.

Even for a nearly ideal spherical aerosol such as iron oxide, we obtained significant differences for parameter estimates from two different observers. While the Quantimet is believed to be a valuable tool for analysis of such samples, it is important to ensure that the size ranges are chosen in such a way that a reasonable number of different size categories are available to satisfactorily estimate distribution parameters. Analysis of results show that the Quantimet as used in the present experiments yielded a significantly higher geometric standard deviation for both the Fe oxide and Pu dioxide aerosols than did observers A and B using the Zeiss particle size analyzer.

Analyses of the Mercer cascade impactor samples taken in connection with these experiments have shown that there is a significant correlation at the $p < 0.05$ level both between aerosol concentration and AMAD and between AMAD and GSD values.

The value of the ESP and TP samples is that they enable one to obtain an

estimate of the actual size of aerosol particles and of their nature, i.e., whether wet or dry, spherical or irregular in shape, discrete or aggregated, etc. The Mercer cascade impactor is advantageous because it directly yields the aerodynamic characteristics of the aerosol. In addition, it is easy to use and the result is more biologically meaningful from the standpoint of estimating alveolar deposition.

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CARCINOGENICITY OF PLUTONIUM DIOXIDE, ASBESTOS, AND
BENZPYRENE IN THE ABDOMINAL CAVITY OF RATS

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²³⁹PuO₂, chrysotile asbestos, and benzpyrene were effective in inducing a large number of abdominal sarcomas and mesotheliomas. The ²³⁹PuO₂ induced tumors originated mostly in the omental region in a dose-dependent manner at radiation doses of from 34,000 rads (65% incidence), 6,300 rads (32% incidence), and 770 rads (17% incidence) delivered to omental tissues. ²³⁸PuO₂ did not induce any mesotheliomas and only 15% and 4% incidence of sarcomas at radiation doses of 31,000 rads and 10,000 rads, respectively, delivered to omental tissues. ²³⁸PuO₂ was spread more evenly throughout the omentum whereas ²³⁹PuO₂ was concentrated within a few "hot spots" of intense alpha activity in omental tissue. Both asbestos and benzpyrene acted in an additive manner when injected with ²³⁹PuO₂, the combination of agents inducing as many sarcomas or mesotheliomas as was obtained by adding tumor incidences from the agents given singly.

The carcinogenicity of PuO₂ particles may be dependent on their site of deposition, particle size, specific activity, dose, and presence of other potentially carcinogenic materials. In a comparative, life-span study, rats were injected into the abdominal cavity with ²³⁹PuO₂ or ²³⁸PuO₂ particles or with a combination of ²³⁹PuO₂ and chrysotile asbestos or benzpyrene to study cocarcinogenic responses.

OBSERVATIONS

Female, Sprague-Dawley CD rats of approximately 70 days of age were given an intraabdominal injection of either ²³⁹PuO₂ (count median diameter, 0.18 μm), ²³⁸PuO₂ (count median diameter, 0.05 μm), 5 mgms 3,4 benzpyrene, or 15 mgms chrysotile asbestos. The amount of Pu injected from the syringe varied by less than 5% from animal to animal in each group as determined by injecting into test vials. The PuO₂ particles were prepared by calcination of Pu oxalate at approximately 350 °C.

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The amounts and combinations of injected materials were as follows with number of animals per group in parenthesis: untreated controls (72), those injected only with saline (36), those injected with 3600 nCi $^{238}\text{PuO}_2$ (36), with 1800 nCi $^{238}\text{PuO}_2$ (24), with 2880 nCi $^{239}\text{PuO}_2$ (36), with 360 nCi $^{239}\text{PuO}_2$ (36), with 70 nCi $^{239}\text{PuO}_2$ (36), with benzpyrene (18), with benzpyrene and 360 nCi $^{239}\text{PuO}_2$ (18), with asbestos (24), and those injected with asbestos and 720 nCi $^{239}\text{PuO}_2$ (24). All materials were injected into the abdominal cavity, suspended in one ml saline. Animals were autopsied at death or when moribund. At necropsy, tissues were taken for determination of plutonium contents and for histoautoradiopathological examination.

Autoradiographic Distribution of Plutonium

The greatest concentration of either $^{239}\text{PuO}_2$ or $^{238}\text{PuO}_2$ occurred in the omentum. Plutonium-239 was greatly concentrated within several densely fibrotic nodules studding the omentum. The nodules were usually 1 to 2 millimeters in diameter. After 6 months, few $^{239}\text{PuO}_2$ particles were found outside these nodules or concentrations of Pu particles. $^{238}\text{PuO}_2$, on the other hand, was more diffusely distributed throughout the omentum and did not exhibit the distinctive "hot spots" of alpha activity as seen with $^{239}\text{PuO}_2$. Alpha stars were rare in mesenteric lymph nodes or in tissues of the gastrointestinal tract. A large number of PuO_2 particles were concentrated within the parasternal lymph node adjacent to the thymus. A

diffuse distribution of alpha stars was seen in the spleen, liver, bone marrow, and lung; comparatively larger PuO_2 particles were seen in the lung and tracheo-bronchial lymph nodes than elsewhere. Occasional alpha activity was found associated with the ovary, uterus, and adrenal glands. Considerably more ^{238}Pu than ^{239}Pu was seen on autoradiograms of skeleton. Generally, a much more diffuse distribution of alpha tracks in tissues was found following $^{238}\text{PuO}_2$ injection than following $^{239}\text{PuO}_2$.

Distribution of Asbestos

The distribution of asbestos was similar to that of $^{239}\text{PuO}_2$ with asbestos fibers being concentrated within fibrous adhesions of the omentum, particularly adjacent to the capsule of the liver, and occasionally within adhesions in the mesenteries. The identification of asbestos fibers in these adhesions was accomplished by electron microscopic examination. Both $^{239}\text{PuO}_2$ and asbestos were distributed within omental adhesions or nodules in animals given both materials together.

Plutonium Content of Tissues

The amount of Pu in organs and tissues is shown in Table 1. About 80% of initially injected ^{239}Pu was retained by rats given 2880 nCi $^{239}\text{PuO}_2$ at a median survival time of 350 days, 67% of 360 nCi $^{239}\text{PuO}_2$ at 470 days, and 30% of 70 nCi $^{239}\text{PuO}_2$ at 580 days. A lesser retention of ^{238}Pu was seen with only 60% of injected ^{238}Pu being found in the terminal body burden at

TABLE 1. Distribution of Intraabdominally Injected Plutonium (Average percent of initially injected dose retained at death.)

Tissue or Organ	Experimental Groups					
	2880 nCi ^{239}PuO	360 nCi $^{239}\text{PuO}_2$	70 nCi $^{239}\text{PuO}_2$	720 nCi $^{239}\text{PuO}_2$ + Asbestos	360 nCi $^{239}\text{PuO}_2$ + Benzpyrene	360 nCi $^{238}\text{PuO}_2$
Liver	11.9	4.1	2.0	8.2	18.9	8.6
Spleen	2.0	1.1	0.4	0.3	1.3	2.0
Kidney	0.05	0.03	--	0.1	0.2	0.1
Visceral Peritoneum	35.0	43.9	15.6	36.0	26.3	18.3
Lung	2.0	0.2	0.3	0.1	1.5	0.1
Thoracic Contents	1.5	1.2	0.8	0.4	4.1	0.8
Skin	0.7	0.3	0.6	0.2	0.6	0.4
Skeleton	1.8	2.7	2.3	1.5	2.6	13.7
Remaining Body Burden	24.2	13.3	7.7	28.1	17.3	16.3
Total Body Burden	79.15	66.83	29.7	74.9	72.8	60.3

a median survival time of 300 days. Injection of $^{239}\text{PuO}_2$ with asbestos or benzpyrene did not influence the retention of ^{239}Pu by the animals.

The greatest concentration of Pu, 18 to 43% of initial dose, occurred in omental and mesenteric tissues collectively called the visceral peritoneum. Over 90% of the $^{239}\text{PuO}_2$ found in the visceral peritoneum was present in the omentum. The liver burden varied with the treatment and survival time of the animals. Little difference in liver burdens was seen between ^{238}Pu and ^{239}Pu . Administration of benzpyrene with $^{239}\text{PuO}_2$ resulted in an increased amount of plutonium transported to the liver. Benzpyrene also increased the translocation of ^{239}Pu into the parasternal lymph nodes and lung. About 14% of injected ^{238}Pu was found in the skeleton at death as compared to only 3% of ^{239}Pu .

Survival and Cause of Death

All groups injected with carcinogens exhibited median survival times that were considerably less than the 760-day median survival time of untreated and saline-injected control animals (Table 2). Groups of rats injected with $^{238}\text{PuO}_2$, 2880 nCi $^{239}\text{PuO}_2$, benzpyrene with or without Pu, and asbestos with Pu survived less than half as long as control animals. Even animals given only 70 nCi $^{239}\text{PuO}_2$ died on the average about 200 days before control animals. The cause of death in many animals given Pu, benzpyrene, or asbestos was malignant abdominal neoplasms. Many rats without life-threatening neoplasms exhibited severe chronic nephritis and fatty necrosis of the liver. Few rats exhibited severe chronic murine pneumonia with its associated bronchiectasis. Bile duct

TABLE 2. Induction of Abdominal Mesotheliomas and Sarcomas by Intraabdominally Injected PuO_2 , Asbestos, and Benzpyrene

Treatment	Median Survival Time (Days) (c)	Amount of ^{239}Pu in		Mean Dose to Omentum (rads)	Incidence of Tumors (%)		
		Visceral	Peritoneum (nCi) (b)		Mesotheliomas	Sarcomas	Total
controls (a)	760	0		---	0	0	0
2880 nCi $^{239}\text{PuO}_2$	350	1,008		33,500	26.5	38.1	64.6
360 nCi $^{239}\text{PuO}_2$	470	142		6,300	10.5	21.0	31.5
70 nCi $^{239}\text{PuO}_2$	580	14		770	2.8	13.9	16.7
15 mg Asbestos	450	0		---	16.7	41.7	58.4
15 mg Asbestos + 720 nCi $^{239}\text{PuO}_2$	340	238		7,700	42.6	33.4	76.0
5 mg Benzpyrene	340	0		---	0	50.0	50.0
5 mg Benzpyrene + 360 nCi $^{239}\text{PuO}_2$	380	134		4,800	5.6	72.3	77.9
3660 nCi $^{238}\text{PuO}_2$	300	1,069		30,500	0	14.6	14.6
1800 nCi $^{238}\text{PuO}_2$	330	331		9,500	0	4.2	4.2

a. 70/100 of the two control groups have been necropsied; half were given saline injection.

b. In tumor-bearing animals only.

c. Data represents survival time for rats after being placed on experiment at 65 to 80 days of age.

epithelial hyperplasia was seen more in animals injected with Pu than in controls. Adrenals of all animals were frequently enlarged, with cortical necrosis and hemorrhaging. Cystic endometrial hyperplasia was seen in several animals. Overall, the cause of death in animals not developing fatal neoplasms appeared similar in most groups. Carcinogen injection appeared to accelerate the normal aging process and the appearance of these pathogenic lesions.

Carcinogenesis - Mesotheliomas

Abdominal mesotheliomas were commonly observed tumors in rats injected with $^{239}\text{PuO}_2$ or asbestos; a 27% inci-

dence of mesothelioma was seen following injection of 2880 nCi $^{239}\text{PuO}_2$ at a mean radiation dose to the omentum of 33,500 rads, an 11% incidence following 360 nCi $^{239}\text{PuO}_2$ at 6300 rads, and a 3% incidence following 70 nCi $^{239}\text{PuO}_2$ at 770 rads to the omentum. A radiation dose of 7700 rads was delivered to omental tissues of rats injected with asbestos and $^{239}\text{PuO}_2$ with the incidence of mesothelioma being 43% as compared to only 16% for animals given only asbestos (Table 2). No mesotheliomas were observed in control animals or in animals injected with benipyrene or $^{238}\text{PuO}_2$. Mesotheliomas appeared earlier in rats given asbestos and $^{239}\text{PuO}_2$ or in rats given larger amounts of $^{239}\text{PuO}_2$ than in

other groups (Figure 1). All but two of the mesotheliomas exhibited large primary growths in the region of the omentum. The tumors were frequently seen proliferating around hot spots of Pu activity in omental adhesions or around asbestos-loaded lesions. Numerous secondary tumor implantations on serosal surfaces in the abdomen and the production of a large bloody ascites volume in the abdominal cavity were characteristic of mesothelioma growth. These tumors were found infiltrating the spleen and liver, lymphatics and muscles of intestinal serosa, and diaphragm.

Carcinogenesis - Sarcomas

The induction of sarcomas was common in rats injected with $^{239}\text{PuO}_2$, as-

bestos, or benzpyrene (Table 2). The sarcomas included fibrosarcomas, osteogenic sarcomas, leiomyosarcomas, rhabdomyosarcomas, liposarcomas, hemangiosarcomas, and one reticulum cell sarcoma in mesenteric lymph node; also included are several undifferentiated sarcomas. The total incidence of sarcoma was influenced by the amount of injected Pu and its combination with benzpyrene or asbestos, with a 38% sarcoma incidence following 2880 nCi $^{239}\text{PuO}_2$, 21% following 360 nCi $^{239}\text{PuO}_2$, and 14% following 70 nCi $^{239}\text{PuO}_2$, as compared to a 50% incidence following benzpyrene and a 72% incidence following benzpyrene and 360 nCi $^{239}\text{PuO}_2$, and as compared to a 42% incidence following asbestos and a 33% incidence following asbestos and 720

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FIGURE 1. Induction of Abdominal Sarcomas and Mesotheliomas by $^{239}\text{PuO}_2$ and Asbestos

nCi $^{239}\text{PuO}_2$. A low incidence of sarcoma induction was seen following injection of $^{238}\text{PuO}_2$, with only 15% incidence being observed at 30,500 rads to the omentum and a 4% incidence following 9500 rads to the omentum (Table 2). As with mesotheliomas, the sarcomas appeared earlier at higher PuO_2 depositions or following combined injection with benzpyrene or asbestos (Figures 1 and 2).

Carcinogenesis - Combined Mesothelioma and Sarcomas

The combined tumor incidence was greater at the higher $^{239}\text{PuO}_2$ depositions with a 65% incidence occurring at 2880 nCi $^{239}\text{PuO}_2$, 32% at 360 nCi $^{239}\text{PuO}_2$, and 17% at 70 nCi $^{239}\text{PuO}_2$. Combining asbestos with $^{239}\text{PuO}_2$ re-

sulted in a combined tumor incidence of 76% as compared to 58% with asbestos alone. Combining benzpyrene with $^{239}\text{PuO}_2$ resulted in a combined tumor incidence of 78% as compared to 50% with benzpyrene alone (Table 2, Figures 1 and 2). Combining $^{239}\text{PuO}_2$ with benzpyrene or asbestos resulted in an additive increase in the production of abdominal mesotheliomas and sarcomas when compared to either agent injected by itself. Both the cumulative incidence of sarcomas and mesotheliomas and their time of appearance were dose dependent.

Carcinogenesis - Osteogenic Sarcomas

Seven osteogenic sarcomas were found in rats injected with PuO_2 . Five of these tumors originated from

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FIGURE 2. Induction of Abdominal Sarcomas and Mesotheliomas by $^{239}\text{PuO}_2$ and Benzpyrene

the femur and six of them had metastasized to the lung. Skeletal radiation doses for animals developing osteogenic sarcomas were 525 rads following 1800 nCi $^{238}\text{PuO}_2$, 1775 rads following 3600 nCi $^{238}\text{PuO}_2$, 160 rads and 170 rads following 2880 nCi $^{239}\text{PuO}_2$, 87 rads following $^{239}\text{PuO}_2$ and asbestos, and 17 rads each for two tumors following benzpyrene and $^{239}\text{PuO}_2$. No osteogenic sarcomas were seen in other groups.

Carcinogenesis - Miscellaneous Groups

Several tumor types were produced by $^{238}\text{PuO}_2$ that were not observed in other groups. These included a tumor of the bile duct epithelium, a melanoma, two undifferentiated carcinomas, and two squamous cell carcinomas in the uterus. Two melanomas were also found in rats injected with asbestos. In addition, a renal adenoma was found at 2880 nCi $^{239}\text{PuO}_2$ and a carcino-sarcoma following $^{239}\text{PuO}_2$ injection.

Comments

PuO_2 particles are concentrated in hot spots within fibrous adhesions of

the omentum; a similar distribution of injected asbestos was also observed. A large number of abdominal sarcomas and mesotheliomas were induced by these omental concentrations of $^{239}\text{PuO}_2$ or asbestos. The $^{239}\text{PuO}_2$ -induced tumors occurred in a dose-dependent manner down to less than 1000 rads delivered to the omental tissues. The smaller sized $^{238}\text{PuO}_2$ particles were concentrated more in the skeleton than was $^{239}\text{PuO}_2$. $^{238}\text{PuO}_2$ was less effective than $^{239}\text{PuO}_2$ as an inducer of abdominal sarcomas or mesotheliomas, even though comparable radiation doses were delivered to visceral peritoneal tissues by the two isotopes. Hot spots of concentrated PuO_2 in parasternal lymph nodes did not result in tumor formation even though their dose distribution was similar to that observed in individual fibrotic omental adhesions. Both benzpyrene and asbestos acted in an additive manner with $^{239}\text{PuO}_2$ in inducing sarcomas and mesotheliomas. There is also a suggestion that combining $^{239}\text{PuO}_2$ with asbestos or benzpyrene may greatly lower the radiation dose to the skeleton required for osteogenic sarcoma induction.

THE DISPOSITION OF INGESTED, INJECTED, AND
INHALED ^{239}Pu CITRATE AND ^{239}Pu NITRATE

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Dogs administered oral, intravenous, or inhaled ^{239}Pu citrate or nitrate were sacrificed from 1 to 100 days after exposure to compare distribution and retention kinetics. Approximately 0.08% of the citrate and 0.005% of the nitrate oral dose was retained after 3 days, distributed mainly between skeleton (50% and 26%) and liver (25% and 26%), respectively. After intravenous administration, the ^{239}Pu dose was retained principally in the liver (32-46% citrate and 21-62% nitrate) and skeleton (29-44% citrate and 13-56% nitrate) during the 100-day postinjection period. Inhaled ^{239}Pu citrate and nitrate were cleared from the blood and excreted with kinetics similar to those described by others for dogs and humans administered intravenous ^{239}Pu citrate. The lung, skeleton, liver, blood, and intestines retained most of the inhaled ^{239}Pu 1 day after exposure. After 100 days, 84-88% of the initial lung burden was retained, principally in the lung, skeleton, and liver. Plutonium was concentrated by lymphatic tissue following all methods of administration at all time intervals. Although the concentration in specific lymph nodes (hepatic, splenic, mediastinal, and tracheobronchial) was among the highest of any tissue analyzed, the total amount deposited in a selection of 12 lymph nodes (~10 grams tissue) was always less than 0.6% of the body burden.

Classical studies of plutonium metabolism in a variety of animals, including man, have employed the citrate complex of Pu administered intravenously. The rationale for the choice of this compound and method of administration has included the assumption that the clearance and translocation kinetics of injected Pu citrate adequately represent the behavior of the solubilized fraction of more "naturally" incorporated Pu. Data presented in this report for ingested, injected, and inhaled Pu citrate and Pu nitrate support the use of injected

Pu citrate as a model to describe the metabolism of soluble plutonium compounds.

OBSERVATIONS

Beagles were administered ^{239}Pu by the various routes employing stock solutions containing 150 μCi Pu/ml in 4% citrate, buffered at pH 3.5 in the case of ^{239}Pu citrate and in 0.27 N HNO_3 for ^{239}Pu nitrate. The citrate and nitrate solutions were 72% and >90% ultrafilterable, respectively, through Visking cellulose tubing.

Twenty-eight beagle dogs 21-24 months of age, average weight approximately 10 kg, were exposed via inhalation to ^{239}Pu citrate or ^{239}Pu nitrate aerosols generated with a Lauterbach generator. Animals were sacrificed in groups of two after 1, 3, 7, 14, 30, 62, and 100 days. Details of the aerosol exposure and preliminary results of the metabolism of inhaled Pu were reported in the 1970 Annual Report.

Six male beagles (three citrate and three nitrate), 19 months of age and 12 kg average weight, were injected in the cephalic vein with 4.35 μCi of ^{239}Pu citrate or 4.97 μCi ^{239}Pu nitrate. One dog from each group was sacrificed at each interval 1, 30, and 100 days after injection.

Two female beagles (one citrate and one nitrate) average weight 11 kg and 99 months of age were administered, by mouth, 580 μCi ^{239}Pu citrate or 644 μCi ^{239}Pu nitrate in gelatin capsules. The dogs were sacrificed 3 days after ^{239}Pu ingestion.

In all cases, the Pu solutions were administered within 3 hr after preparation employing the concentrated solution (150 $\mu\text{Ci}/\text{ml}$) without further dilution. The animals were housed in metabolism cages where either daily or pooled urine and feces samples were collected. The dogs were sacrificed by exsanguination under sodium pentobarbital anesthesia and subjected to detailed necropsy. Tissue and excreta samples were analyzed for Pu using a scintillation counting technique.

Plutonium was cleared from the blood with fairly similar kinetics following inhalation and intravenous

administration of the citrate and nitrate salts (Figure 1). This suggests that the mechanisms of Pu transport and binding in blood may be the same for these modes of administration. The relatively prolonged clearance observed with Pu nitrate and inhaled Pu citrate is likely a consequence of the continuing mobilization of Pu from the reservoirs in lung and liver. It is rather remarkable that less than order-of-magnitude differences were generally observed among the studies in Figure 1 considering the different chemical compounds employed and the differences in species and method of administration.

FIGURE 1. Blood Clearance of ^{239}Pu Administered as ^{239}Pu Citrate or ^{239}Pu Nitrate

The pattern of urinary excretion was also closely similar for the inhaled and injected Pu compounds (Figure 2). The actual data points shown for two dogs injected intravenously varied considerably from day to day which is not unusual for this type of

study, but the reason is not readily apparent. Similar results were observed for the summed daily urinary and fecal excretion (Figure 3). Values for the inhalation dogs were highest (about 10-fold higher than the

FIGURE 2. Urinary Excretion of ^{239}Pu Administered as ^{239}Pu Citrate or ^{239}Pu Nitrate

daily urinary excretion shown in Figure 2) probably because Pu was continually cleared via the ciliated epithelium and was subsequently swallowed and eliminated by way of the gastrointestinal tract.

The distribution of Pu in tissues was significantly influenced by the method of administration as shown in Table 1. About 0.08% of the Pu citrate and 0.005% of the Pu nitrate administered orally was absorbed from

FIGURE 3. Total Excretion of ^{239}Pu Administered as ^{239}Pu Citrate or ^{239}Pu Nitrate

TABLE 1. Distribution of Plutonium in Beagle Dogs After Administration of the Citrate and Nitrate by Various Routes

Route of Administration	Days After Administration ^(b)	Percent Administered Dose ^(a)						Total Animal	
		Lung		Liver		Skeleton		Pu Citrate	Pu Nitrate
Oral	3 (1)	0.0006	0.00007	0.02	0.0013	0.04	0.0013	0.083	0.005
Intravenous	1 (1)	1.8	2.0	32	62	29	13	114	115
	30 (1) ^(c)	0.12	0.52	6.2	21	44	56	68	91
	100 (1)	0.77	0.18	46	35	29	39	86	82
Inhalation	1 (2)	74	88	1.4	0.32	6	1.8	99	100
	3 (2)	46	86	8.8	1.2	25	5.7	99	100
	7 (2)	42	73	5.1	3.8	40	12	99	98
	14 (2) ^(d)	38	42	14	2.6	32	27	93	88
	30 (2)	37	32	5.3	9	38	43	86	91
	62 (2)	26	36	14	8.8	44	36	90	86
	100 (2)	29	41	16	10	38	28	88	84

a. Dose for inhalation dogs was computed as the amount recovered less the amount excreted in feces during the first 6 days.

b. Number of dogs for each compound in parenthesis.

c. Approximately one-half of the injected Pu citrate was recovered at the injection site. The dose was adjusted for this loss.

d. Only one dog in 14-day Pu citrate group.

the gastrointestinal tract and deposited mainly in the skeleton and liver. Intravenous Pu citrate and nitrate were initially deposited more extensively in the liver than in skeleton; however, this relationship tended to vary at the later time intervals. The distribution picture in the dog sacrificed 30 days after Pu citrate is complicated by the retention of about one-half the dose in tissues adjacent to the injection site. A large fraction of the inhaled Pu citrate was retained in the lung at all time intervals. That fraction translocated from the lung was distributed between skeleton and liver not unlike the pattern for oral ingestion or after intravenous injection of monomeric plutonium citrate as reported by others (Stover et al., Rad. Res. 10, 130, 1959).

Both the inhaled and injected dose were retained to about the same extent over the 100-day experimental period.

It was an unexpected finding that inhaled Pu citrate was retained so tenaciously, i.e., about 30% of the estimated initial lung burden was found in lung after 100 days. This may be due in part to the relatively high percentage of polymerized Pu in the solutions (about 25% was not ultrafilterable) but is more probably to be expected as a consequence of hydrolysis and polymerization in the lung environment since about 40% of the inhaled Pu nitrate was also retained. In either case, a high concentration of Pu in the lung was characteristic after inhalation exposure as shown in Table 2. The concentration of Pu in turbinates (this sample included

TABLE 2. Concentration of Plutonium in Organs of the Beagle Dog After Administration of the Citrate and Nitrate by Various Routes

Route of Administration	Days After Administration ^(b)	²³⁹ Pu Concentration (% dose/gram X 10 ³) ^(a)					
		Lung		Turbinates		Lymph Nodes ^(c)	
		Pu Citrate	Pu Nitrate	Pu Citrate	Pu Nitrate	Pu Citrate	Pu Nitrate
Oral	3 (1)	0.008	0.001	0.014	0.0023	0.033 (0.013-0.124)	0.0042 (0.0014-0.19)
Intravenous	1 (1)	24	30	25	22	54 (6-158)	29 (10-66)
	30 (1) ^(d)	2	11	58	60	32 (7-1072)	30 (6-45)
	100 (1)	9	2.3	25	27	34 (19-106)	20 (6-82)
Inhalation	1 (2)	1048	1482	113	137	12 (2-328)	10 (2-172)
	3 (2)	689	1624	138	116	21 (3-228)	14 (2-313)
	7 (2)	630	1011	247	36	18 (3-312)	15 (2-256)
	14 (2) ^(e)	485	633	168	96	26 (7-230)	30 (8-492)
	30 (2)	617	394	161	100	48 (9-848)	36 (7-516)
	62 (2)	459	440	53	134	53 (5-1176)	61 (8-606)
	100 (2)	503	614	129	67	99 (2-1278)	120 (6-4465)

a. Dose for inhalation dogs was the amount recovered less the amount excreted in feces during the first 6 days.

b. Number of dogs in parentheses.

c. Values are the average of 12 different lymph nodes with range in parentheses.

d. Approximately 50% of the dose was recovered at the injection site of the Pu citrate dog. The dose was adjusted for this loss.

e. Only one dog in 14-day Pu citrate group.

all soft tissue and bone that could be scraped from the nasal cavity) exceeded that in bone or liver. Although the early deposition can be explained by physical trapping during inhalation, the retention at later time intervals as well as the high concentration after intravenous injection implies that plutonium is selectively deposited in turbinates possibly because of the well developed vascularization of this tissue.

It is noteworthy that lymph nodes concentrated Pu following all three routes of administration. At all time intervals, the concentration in specific nodes was among the highest of any tissue analyzed. The values for a selection of 12 nodes taken from different anatomical locations ranged

over several orders of magnitude as indicated in parenthesis in Table 2. Either the splenic, hepatic, mediastinal, or tracheobronchial nodes accounted for the highest concentrations within the range of values. The lymph nodes which concentrated the maximum amount of plutonium were always 'closely related anatomically to major sites of Pu deposition. It was noted after intravenous injection that the prescapular nodes immediately anterior to the injection site contained exceptionally large amounts of Pu. The concentration in these nodes was not included in Table 2. The dog sacrificed 30 days after intravenous injection gave anomalous values for tissue distribution because of a poor

injection which left about one-half of the dose in the perivascular tissue at the injection site. Approximately 8% of the dose was recovered in the prescapular nodes of this dog.

CONCLUSIONS

Although the number of animals employed in this study was quite limited, the results support the use of intravenous Pu citrate as a model system to describe the metabolism of Pu absorbed by less direct routes. Intravenous Pu citrate appeared to show blood clearance, deposition, and excretion kinetics that were fairly representative of incorporation by routes of entry more commonly encountered in occupational exposure. Inhalation exposure, of course, led to greater relative retention in the lung. The distribution of intravenous Pu citrate is itself somewhat unpredictable, being influenced by the physical-chemical variables of the injection solutions. Plutonium levels in blood and urine, however, were surprisingly little affected by the large differences in tissue distribution. Evaluation of human exposures to soluble Pu, based on analyses for Pu in urine, may not be too inaccurate when interpreted on the basis of human studies with injected Pu citrate. It seems clear that Pu nitrate should also be considered a "soluble" compound in

this connotation since inhaled citrate and nitrate salts of Pu are translocated and redistributed similarly.

The tendency for Pu to concentrate in lymph nodes emphasized the role of the lymphatic system in normal Pu clearance and the potential for Pu to concentrate in relatively small tissue volumes. The accumulation of Pu in regional lymph nodes following subcutaneous injection was demonstrated in the Pu citrate dog sacrificed 30 days after intravenous injection. It seems probable, therefore, that the highest radiation doses may be observed in lymph nodes following industrial exposure to Pu. Although the significance of this dose to only a small fraction of the total lymphatic system is not clearly understood in terms of the long-term effects of Pu incorporation, it is of interest that tumors involving lymph nodes have been described in dogs following exposure to Pu by both inhalation and injection. Further investigations in particular low-level, long-term studies are needed to establish the relative effectiveness of radiation to the skeleton, liver, lung, and lymph nodes. At the present time, the well established effects in bone, liver, and lung would seem to require first consideration for these tissues as critical organs for radiation hazard considerations.

DISTRIBUTION AND CLEARANCE OF INHALED
SAMARIUM AND PROMETHIUM OXIDES IN RATS

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$^{145}\text{Sm}_2\text{O}_3$ and $^{143}\text{Pm}_2\text{O}_3$ were translocated from the lung and deposited in tissues in a similar manner, suggesting that Sm is a suitable carrier for Pm. The rates of translocation and excretion suggest that the oxides prepared by calcining at 750 °C are moderately soluble in the rat lung.

Samarium is being investigated as a potential carrier for Pm as part of a study designed to develop a method for determining the body burden of $^{147}\text{Pm}_2\text{O}_3$ in man. Preliminary results in rats, reported in the 1970 Annual Report, suggest that ^{147}Pm and ^{145}Sm are metabolized similarly when injected as soluble citrate complexes. The present report extends the rat studies to include investigations with insoluble compounds administered by the inhalation route.

PROCEDURE

Thirty-six adult female rats were exposed for 1 hr to an aerosol (AMAD

3.37 μm σg 1.57) generated from a water suspension containing a mixture of the oxides of ^{145}Sm (15 $\mu\text{Ci/ml}$), ^{143}Pm (5 $\mu\text{Ci/ml}$), and nonradioactive Sm (15 mg/ml) as carrier. The oxides were prepared by calcining the metal oxalate salts at 750 °C, following their precipitation with oxalic acid from a HCl solution containing all three nuclides. The animals were sacrificed in three groups of 12 immediately after exposure, after 14 days, and after 30 days. The amount of ^{145}Sm and ^{143}Pm was determined in lung, liver, kidney, muscle, skeleton, pelt, head, urine, and feces using a coincidence gamma scintillation counting technique. The counting system could detect 3.5 pCi of either radio-nuclide as the lower limit.

RESULTS AND DISCUSSION

Rats exposed for 1 hr to aerosols containing 16.8 nCi $^{145}\text{Sm}/\text{l}$ and 4.8 nCi $^{143}\text{Pm}/\text{l}$ retained, on the average,

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3.87 nCi ^{145}Sm and 1.02 nCi ^{143}Pm in the lung when sacrificed immediately after exposure (Table 1). The initial lung burden varied six-fold among animals in this group either because of variations in the aerosol concentration within the exposure chamber or because of physiological differences which influenced deposition in the respiratory tract. Values for lung retention of ^{145}Sm and ^{143}Pm at the later intervals also ranged seven- to eleven-fold, suggesting that the variations in early deposition were associated, at least in part, with areas of the lung that exhibit relatively slow clearance kinetics. Although the amount of Sm carrier was not measured directly in this study, it was assumed to follow the ^{145}Sm tracer and was estimated from the specific activity (1 nCi $^{145}\text{Sm}/\mu\text{g Sm}$) of the starting solution. On this basis the aerosol concentration was 16.8 $\mu\text{g Sm/l}$ and the initial lung burden amounted to 3.87 μg . An equivalent lung burden in man would be about 2.5 mg which is within the

range of values that might be considered acceptable for future studies in humans using samarium carrier.

At all time intervals, the relative amounts of ^{145}Sm and ^{143}Pm in lung agreed reasonably well with their relative concentrations in the aerosol, confirming that neither radionuclide was selectively deposited or retained in the lung. Retention in other tissues and the pattern of excretion (Table 2) were generally quite similar except for the deposition in kidneys, where the discrepancy may be due to errors associated with low counting rates.

It is of interest that about 10% of the estimated initial lung burden was recovered in tissues excluding lung, pelt, and head at the 14-day interval. Assuming that the radioactivity in urine was translocated primarily from the lung (<0.01% gut absorption of Sm and Pm), one must conclude that the inhaled oxides were unusually well absorbed. This is also indicated by the continued buildup of ^{145}Sm and

TABLE 1. Aerosol Concentration and Lung Burden of ^{145}Sm , ^{143}Pm , and Carrier Sm (Values are averages of three aerosol samples or 12 animals with range in parenthesis.)

	Aerosol	Lung Burden		
		Initial	14 Days	30 Days
^{145}Sm	16.8 nCi/l (13.8-20.1)	3.87 nCi (1.38-8.87)	2.39 nCi (0.70-7.73)	1.53 nCi (0.49-3.86)
^{143}Pm	4.8 nCi/l (3.79-5.87)	1.02 nCi (0.36-2.38)	0.67 nCi (0.21-2.24)	0.43 nCi (0.16-1.02)
Sm Carrier	16.8 $\mu\text{g/l}$ (13.8-20.1)	3.87 μg (1.38-8.87)	2.39 μg (0.70-7.73)	1.53 μg (0.49-3.86)

^{143}Pm in liver and skeleton throughout the 30 days following exposure. It should be noted, however, that the lung burden decreased by only 20% of the initial burden during the 14- to 30-day postexposure period while the increase in excreta and tissues during this same time interval was about twice this amount. Such errors in material balance are likely due to the averaging of values from animals that ranged as much as ten-fold in deposition and retention of ^{145}Sm and ^{143}Pm .

The excretion of ^{145}Sm in urine and feces, shown in Figure 1, is also representative of the excretion curves for ^{143}Pm . The large amount of ^{145}Sm associated with early excretion via feces is known to be a function of the aerosol particle size and may

have been augmented significantly by ^{145}Sm cleared from the lung during the 1-hr exposure period. It is estimated from these data that only 2.5% of the total body burden was deposited in the lung immediately after exposure. Surprisingly large amounts of ^{145}Sm were excreted in urine, although the oxides of Sm and Pm are classified, along with oxides of the actinide elements, as Class Y compounds by the ICRP. Contamination of the urine with feces cannot be excluded as a possibility, particularly at the early time intervals; however, this seems unlikely since the daily urine values at later times were consistently an order of magnitude or more higher than comparable values for soluble or insoluble Pu compounds. The high level of urinary excretion implies that the inhaled oxides were at least moderately soluble. This is consistent with the amount of the radionuclides translocated to tissues (Table 2) and the half-life of clearance from the lung between 14 and 30 days postexposure, estimated to be approximately 25 days.

CONCLUSIONS

The metabolism of inhaled $^{145}\text{Sm}_2\text{O}_3$ and $^{143}\text{Pm}_2\text{O}_3$ appears to be similar in the rat, suggesting that Sm would be a suitable substitute for Pm in proposed studies in man. Oxides of Pm and Sm prepared by calcining at 750°C are moderately soluble in the rat. About 10% of the initial lung burden was translocated to other tissues while possibly an even larger amount was excreted in urine. The

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FIGURE 1. Excretion of ^{145}Sm in Urine and Feces

TABLE 2. Disposition of Inhaled $^{145}\text{Sm}_2\text{O}_3$ and $^{143}\text{Pm}_2\text{O}_3$

	% Initial Lung Burden ^(a)			
	14 Days		30 Days	
	^{145}Sm	^{143}Pm	^{145}Sm	^{143}Pm
Lung	62	66	40	42
Liver	2.9	3.3	4.1	5.3
Kidneys	0.28	0.35	0.13	0.37
Pelt	12	14	3.9	4.6
Remaining Soft Tissue	2.5	3.4	NM	NM
Head	1.2	1.6	2.6	2.6
Remaining Skeleton	3.4	3.6	5.5	5.1
Accumulated Excreta				
Urine	54	51	60	58
Feces	4010	3880	4040	3905

NM = not measured

a. Initial lung burdens and ranges were 3.87 (1.38-8.87) nCi ^{145}Sm and 1.02 (0.36-2.38) nCi ^{143}Pm determined by sacrificing animals immediately after exposure.

clearance half-time from lung ($T_{1/2}$ = 25 days from 14 to 30 days postexposure) and the extent of translocation to nonpulmonary tissues suggests that

oxides of Sm and Pm should be classified in Class W according to the ICRP Committee II nomenclature.

REMOVAL OF INHALED $^{239}\text{PuO}_2$ FROM BEAGLE DOGS

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*Beagle dogs were exposed to $^{239}\text{PuO}_2$ aerosols and treated with DTPA,** Phenergan,† DTPA plus Phenergan, or pulmonary lavage to determine the efficacy of these treatments in removing inhaled plutonium. Lavage was accomplished by using a double lumen Carlen's broncho-spirometry catheter, lavaging the right lung only with 0.9% NaCl, leaving the left lung to serve as control. Treatment continued on all groups for approximately 20 weeks. The drug-treated groups retained essentially the same amount of plutonium as the control group, while the lavage animals had a reduction of 50% in the lavaged lung. The final burden of the lavaged group was 67% of the initial alveolar burden compared to 93% for the controls and 92% for the other treated animals. At the present time pulmonary lavage is the most effective method to remove inhaled $^{239}\text{PuO}_2$ from the lung.*

Beagle dogs were exposed to $^{239}\text{PuO}_2$ aerosols and treated with DTPA, Phenergan, DTPA plus phenergan, or pulmonary lavage to determine the efficacy of these treatments in removing inhaled plutonium. DTPA is used for removal of "soluble" plutonium. Although generally not considered to be useful for removal of insoluble plutonium, DTPA was included in this experiment to compare with the other removal procedures. Phenergan was

previously shown to reduce the plutonium lung burden in rats, but there was an increased retention in other tissues (Annual Report, 1969). These results suggested that Phenergan plus DTPA might prove to be a beneficial therapeutic agent. Pulmonary lavage has been reported to be an effective means of removing inhaled radioactive particles by several investigators. (1,2,3)

OBSERVATIONS

The experimental design is shown in Table 1. Twenty, 8-13 kg, 14- to 21-month-old beagle dogs were exposed 7 to 10 min to an aerosol of $^{239}\text{PuO}_2$ (calcined for 2 hr at 750 °C, AMAD

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** *Trisodium calcium diethylenetriaminepentaacetic acid*

† *Phenergan^(R) promethazine hydrochloride*

TABLE 1. Experimental Design

<u>Exposure</u>	<u>Treatment</u>	<u>No. of Dogs</u>	<u>Days to Sacrifice</u>
None	None	4	---
$^{239}\text{PuO}_2$	None	4	141
$^{239}\text{PuO}_2$	Weekly lavage of right lung	4	150
$^{239}\text{PuO}_2$	Daily Phenergan (oral - 50 mg)	4	142
$^{239}\text{PuO}_2$	Twice weekly DTPA (I.V. - 100 mg)	4	138
$^{239}\text{PuO}_2$	DTPA and Phenergan	4	134

2.5, GSD 1.8) and divided into five groups of four dogs each: controls, lavage, DTPA, Phenergan, and DTPA plus Phenergan. Treatment was started immediately after exposure on all groups, except the lavaged group which started on the eighth post-exposure day, and continued for approximately 20 weeks. All the dogs were housed in metabolism cages and excreta collected for the duration of the experiment.

The lavage dogs were anesthetized with Na Pentathol and a double lumen Carlen's bronchspirometry catheter was placed in the trachea and bronchi to achieve complete separation of the airways to the right and left lung. Oxygen was administered for 5 min through the catheter to the right lung. It was then closed for 10 min to allow for degassing before the lavage was started while oxygen was administered to the left lung for the remainder of the lavage procedure. Two hundred ml 0.9% NaCl was introduced through the catheter into the

right lung. Of the first 200 ml, about 75% was recovered by postural drainage; but with the next two succeeding 200 ml lavages, nearly all of the lavage fluid was recovered. After the third lavage, oxygen was again administered to the right lung until the dog started to regain consciousness, at which time the Carlen's tube was removed and the dog placed in a metabolism cage and closely observed until fully ambulatory. Weekly lavage continued for 19 weeks, lavaging the right lung only. By the nineteenth week, less than 0.4% of the estimated lung burden was being removed from each dog; and the experiment was terminated.

The total quantity of plutonium deposited in each dog was determined from analyses of all urine, feces, lavage samples, and all tissues by scintillation counting. Whole-body clearance curves were constructed for each dog by subtracting cumulative excretion and lavage values from the total quantity of plutonium deposited.

Figure 1 shows the mean whole-body retention curves for the five groups. Since the early clearance phase was complete in all dogs by 8 days post-exposure, the total body burden 8 days postexposure was used as the estimated fraction of plutonium that was unavailable for early clearance. This fraction was assumed to have been deposited in the alveoli and accounted for the long-term retention of plutonium in the dogs. Since 99% of the final plutonium body burden was in the lungs and thoracic lymph nodes and 99% of the excreted plutonium was in the feces, it is doubtful that non-alveolar deposited plutonium contributed significantly to the 8-day post-exposure body burden.

The estimated percent of the total deposited plutonium that was deposited in the alveoli ranged from 73% to 98% in the plutonium control and plutonium + lavage group which did not receive any treatment until 8 days postexposure (Table 2). Although the DTPA, Phenergan, and DTPA + Phenergan treatments started on the day of exposure may have had some effect on early alveolar clearance, the effect, if any, on the fraction retained in these groups 8 days postexposure was not different from the plutonium controls or plutonium + lavage groups.

The slope of the whole-body retention curves between 8 and 150 days postexposure for the three drug-treated groups are not significantly different from the controls (Figure 1).

FIGURE 1. Whole-body Retention of Inhaled $^{239}\text{PuO}_2$
(Mean of four dogs per group)

TABLE 2. Disposition of Inhaled $^{239}\text{PuO}_2$

Treatment (Dog)	Initial Body Burden ^{239}Pu (μCi)	Initial Alveolar Burden(b) (% of Initial Body Burden)	Final Body Burden (%Initial Alveolar Burden)	Final Lung Burden (%Initial Alveolar Burden)	Whole-Body Retention Half-time(c) (days)	Lung Retention Half-time(c) (days)
Control-1	1.7	98	99	93	8210	1280
2	2.0	90	98	96	4870	2100
3	1.0	92	96	93	2690	1280
4	1.6	95	97	92	3770	1230
Mean (a)	1.6 $\pm$ 0.6	94 $\pm$ 6	98 $\pm$ 2	93 $\pm$ 2	4880 $\pm$ 3810	1480 $\pm$ 670
Lavage- 1	2.0	73	78	73	420	330
2	1.1	86	63	59	220	200
3	2.1	93	68	65	270	240
4	1.8	85	78	69	410	280
Mean (a)	1.8 $\pm$ 0.7	84 $\pm$ 13	72 $\pm$ 12	67 $\pm$ 10	330 $\pm$ 160	260 $\pm$ 90
OIPA- 1	4.2	93	99	93	7220	1220
2	5.1	98	98	89	5840	820
3	2.8	84	96	90	2320	920
4	3.9	91	97	92	3540	1180
Mean (a)	4.0 $\pm$ 1.5	91 $\pm$ 9	98 $\pm$ 2	91 $\pm$ 3	4730 $\pm$ 3520	1030 $\pm$ 310
Phenergan-1	4.6	93	98	90	4380	950
2	3.2	80	97	90	2910	890
3	4.7	96	98	87	5260	680
4	2.3	96	98	92	5930	1140
Mean (a)	3.7 $\pm$ 1.9	91 $\pm$ 12	98 $\pm$ 1	90 $\pm$ 3	4620 $\pm$ 2080	920 $\pm$ 300
DTPA + Phenergan 1	3.6	82	98	92	3930	1120
2	2.8	88	97	90	2520	840
3	2.9	77	95	92	1770	1070
4	2.9	94	98	90	4450	910
Mean (a)	3.1 $\pm$ 0.6	85 $\pm$ 11	97 $\pm$ 2	91 $\pm$ 2	3160 $\pm$ 1970	990 $\pm$ 210

a. Mean of four dogs $\pm$ 95% confidence interval around the mean.

b. Estimated from 8 days postexposure body burden.

c. Calculated using initial alveolar burden.

In Table 2, the amount of plutonium found in the body and in the lung at necropsy is expressed as a fraction of the amount initially deposited in the alveoli. Assuming the clearance of plutonium was exponential with time, whole-body and lung retention half-times were obtained for each dog for convenience of comparing treatment groups. There was no evidence that percentage alveolar deposition or retention parameters of the DTPA, Phenergan, or DTPA + Phenergan groups were significantly different from the plutonium control group. The lavage group lost approximately 28% of their Pu body burden from day

8 to 150, while the other groups excreted less than 3% over the same time period (Figure 1). The whole-body retention and lung retention half-time for the lavage group was very sharply reduced during the treatment period (Table 2).

The amount of plutonium obtained in the lavage fluid decreased with time postexposure. About 50% of the plutonium removed by lavage was obtained in the first 5 weeks and 85% by the eleventh week (Figure 2). Table 3 shows that about 27% of the initial alveolar burden was removed in the lavage fluid while about 2% was excreted. The fraction of initial

FIGURE 2. Cumulative Percent of Total Plutonium Removed in the Lavage Fluid

TABLE 3. Plutonium Removal by Pulmonary Lavage

Group	Treatment	Initial Alveolar Burden (nCi) ^(b)	Total ²³⁹ Pu Removed in Lavage Fluid (nCi)	% of Initial Alveolar Burden in Lavage Fluid	Total ²³⁹ Pu Removed in Excreta ^(c) (nCi)	% of Initial Alveolar Burden Excreted
1	Lavage	1500	304	20.3	26	1.7
2	Lavage	958	327	34.1	31	3.2
3	Lavage	1931	594	30.8	26	1.3
4	Lavage	1500	313	20.9	24	1.6
Mean ^(a)				26.5 ± 11.1		2.0 ± 1.4
1	Control	1702	0	0	20	1.2
2	Control	1761	0	0	35	2.0
3	Control	948	0	0	33	3.5
4	Control	1551	0	0	39	2.5
Mean ^(a)						2.3 ± 1.5

a. Mean of four dogs ±95% confidence interval around the mean.

b. Estimated from 8 days postexposure body burden.

c. Estimated by subtracting the difference between the initial amount of Pu deposited in the dog and the estimated initial Pu in the alveoli from the total amount of Pu excreted.

alveolar burden excreted by the lavaged dogs was not significantly different from that excreted by the control group, suggesting that nearly all of the decreased Pu retention in the lavage dogs was due to plutonium removed in the lavage fluid. We had expected the lavage group to show more excretion due to an increased availability of plutonium not removed with the lavage fluid for ciliary clearance.

Table 4 compares the plutonium concentration per gram of lung tissue in the right (lavaged) and left lung at death. The mean ratio of the right lung to left lung of all dogs, excluding the lavage group, was 1.03 while the mean ratio of the lavage group was 0.50. Assuming equal success lavaging both lungs, one could predict that about 50% of the initial alveolar lung burden could be removed by pulmonary lavage. Evaluation of hematology, clinical chemistry, histopathology, and autoradiography collected on these dogs is in progress to determine the biological effects of the pulmonary lavage in these studies.

The distribution of plutonium in the tissues at death is shown in Table 5. None of the treatments appeared to have a significant effect on the Pu distribution with about 94% of the final Pu body burden in the lung and 5% in the tracheobronchial and mediastinal lymph nodes.

CONCLUSION

DTPA, Phenergan, and DTPA + Phenergan were not effective in re-

TABLE 4. ²³⁹Pu Concentration in Lungs at Death (a)

Dog	Treatment	Right Lung ($\mu\text{Ci/gm}$)	Left Lung ($\mu\text{Ci/gm}$)	Ratio RL/LL
1	Control	0.027	0.026	1.04
2		0.025	0.020	1.25
3		0.012	0.012	1.03
4		0.027	0.026	1.02
Mean				1.08
1	Lavage	0.012	0.026	0.46
2		0.004	0.009	0.46
3		0.009	0.022	0.39
4		0.011	0.016	0.70
Mean				0.50
1	DIPA	0.042	0.047	0.89
2		0.059	0.052	1.13
3		0.032	0.032	1.02
4		0.038	0.036	1.04
Mean				1.02
1	Phenergan	0.042	0.033	1.26
2		0.040	0.039	1.02
3		0.044	0.048	0.93
4		0.028	0.032	0.88
Mean				1.02
1	DIPA + Phenergan	0.035	0.036	0.96
2		0.032	0.034	1.04
3		0.021	0.022	0.93
4		0.036	0.033	1.07
Mean				1.00

Average for all groups except lavaged group 1.03

a. Based on wet weight of lungs at necropsy.

moving inhaled ²³⁹PuO₂ from the lung. Pulmonary lavage removed about 50% of the plutonium from the lavaged lung. At the present time, pulmonary lavage is the only effective method to remove inhaled insoluble plutonium from the lung. Studies on the use of other lavage fluids and the time and regimen of treatment are in progress to further evaluate the efficacy of this therapy procedure.

TABLE 5. Distribution of **Inhaled $^{239}\text{PuO}_2$** in Tissues

Treatment (Dog)	Postexposure (days)	Final Body Burden (μCi)	^{239}Pu in Tissue (% of Final Body Burden)		
			Lung	Lymph Nodes ^(b)	All Others
Control-1	141	1.7	94	6	<1
2		1.7	97	3	<1
3		0.9	96	4	<1
4		1.5	95	5	<1
Mean ^(a)		1.5 $\pm$ 0.6	96 $\pm$ 3	4 $\pm$ 2	
Lavage-1	150	1.2	94	6	<1
2		0.6	95	5	<1
3		1.3	95	5	<1
4		1.2	90	10	<1
Mean ^(a)		1.1 $\pm$ 0.5	93 $\pm$ 4	6 $\pm$ 4	
DIPA-1	142	3.9	94	6	<1
2		4.9	91	9	<1
3		2.3	94	6	<1
4		3.3	95	5	<1
Mean ^(a)		3.6 $\pm$ 1.7	93 $\pm$ 3	7 $\pm$ 3	
Phenergan-1	138	4.2	92	7	<1
2		2.5	93	7	<1
3		4.4	88	11	<1
4		2.2	93	7	<1
Mean ^(a)		3.3 $\pm$ 1.8	92 $\pm$ 4	8 $\pm$ 3	
DIPA + 1	134	2.9	94	6	<1
Phenergan 2		2.4	93	7	<1
3		2.1	97	3	<1
4		2.7	92	8	<1
Mean ^(a)		2.5 $\pm$ 0.6	94 $\pm$ 3	6 $\pm$ 3	

a. Mean of four dogs $\pm$ 95% confidence interval around mean.

b. Tracheobronchial and mediastinal lymph nodes.

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ACUTE TOXICITY OF DTPA ADMINISTERED INTRATRACHEALLY

Investigators:

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Technical Assistance:

W. G. Morrow

Rats tolerated a total of 200 mg of CaNa_3 - or ZnNa_3 -DTPA when the dose was administered intratracheally as a series of 50 mg aliquots, one given every other day. Doses of 75 mg or greater were lethal to some animals. Moderate to severe inflammation of pulmonary tissues was seen as the major histopathologic effect of intratracheal DTPA. These changes were of a transient nature and repaired within 2 to 4 weeks after drug treatment ceased.

DTPA (diethylenetriaminepentaacetic acid) therapy has been successfully administered to people by aerosol inhalation as a means of expediting drug treatment while avoiding the anxiety associated with repeated intravenous injections. Assuming the inhalation route is the treatment of choice for industrial exposures to plutonium, further knowledge of the biological effect of DTPA in the lung is needed. To our knowledge, no investigation has yet been made of the acute or chronic toxicity of inhaled DTPA. The present study was designed to provide such information by exposing rats to intratracheally instilled DTPA, inhaled DTPA aerosols, and a combination of repeated inhaled DTPA treatments following inhalation exposure to $\text{Pu}(\text{NO}_3)_4$. Preliminary results are reported here for the acute toxicity of intratracheally instilled DTPA.

OBSERVATIONS

Female rats averaging 290 ± 10 grams were anesthetized with halothane and intratracheally instilled with 0.45 ml of solutions of osmotic pressure 1650 mOs, according to a procedure described elsewhere in this report. Animals administered 50 mg of CaNa_3 - or ZnNa_3 -DTPA every other day for a total of four instillations tolerated the dosage well (Figure 1). A dose of 75 mg administered in the same fashion was lethal to one of ten rats given CaNa_3 -DTPA. Only three instillations were administered at the higher dose levels because it was apparent all animals would die if the drug treatment was continued. Control animals administered NaCl survived the treatment without deaths; however, Na_3 citrate was lethal to two of ten rats after the second instillation. Animals died within minutes or a few

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FIGURE 1. Acute Toxicity of Intratracheally Instilled $\text{CaNa}_3\text{-DTPA}$ and $\text{ZnNa}_3\text{-DTPA}$ in Rats (Drugs were administered every other day in a 0.45 ml volume osmotic pressure 1650 mO's, ten rats per group except for 87.5 mg $\text{ZnNa}_3\text{-DTPA}$ where only eight were used.)

hours after the final instillation due to suffocation induced by the instilled solutions.

Animals were sacrificed for histopathologic examination immediately after the final treatment and after periods of recovery of 14 days and 30 days following the final instillation. Although lung, liver, spleen, kidney, and gastrointestinal tract were examined, only the lesions in the lung were thought to be due to the drug treatment.

Rats receiving four treatments of 50 mg $\text{CaNa}_3\text{-DTPA}$ and sacrificed immediately following the last treatment showed only minimal histologic effects. A few small focal areas of fibroblastic activity were seen scattered throughout the lungs (Figure 2).

These foci most often were adjacent to air passageways, vessels, and in-

FIGURE 2. Lung of Rat Sacrificed Immediately After a Series of Four Intratracheal Instillations with 50 mg $\text{CaNa}_3\text{-DTPA}$ [A small focal fibroblastic proliferation (A) is located near a terminal bronchiole (B). 200X]

terlobular septa. The fibroblastic elements showed little collagenization. Hyperplasia and hypertrophy of

alveolar lining cells were apparent in these lungs. This, along with infiltration of alveolar septa with mononuclear cells and slight fluid accumulation, caused moderate septal thickening. Alveoli were distended and occasional ruptured septa were present. Macrophages were accumulated along the septa within the alveolar spaces and sometimes were free within the spaces.

At the 75 mg and 87.5 mg level of $\text{CaNa}_3\text{-DTPA}$, the histopathologic changes were quite similar in detail to those described above but with noticeable increase in number and size of fibroblastic foci (Figure 3). No obvious differences were observed between the severity of lesions in rats which received 75 mg and 87.5 mg $\text{CaNa}_3\text{-DTPA}$.

The rats which received 100 mg and those which received 112.5 mg of

$\text{CaNa}_3\text{-DTPA}$ had lung lesions of much increased severity relative to those which received lower dosages. The lesions in these groups were patchy in distribution, affecting all lobes of the lungs, and consisted of near total consolidation. This consolidation was produced by proliferations of fibroblastic tissue, histiocytes, and epithelial tissue. Bronchiolization (cuboidal metaplasia) of alveolar lining epithelium was present along with alveolar cell hyperplasia, mononuclear cell infiltration of septa and perivascular areas, and fibroblastic activity (see Figure 4). In areas of the lesions, edema fluid has flooded alveolar spaces.

In rats allowed 2 weeks of recovery before euthanasia, only minimal lesions were present in the lungs.

FIGURE 3. Lung of Rat which Received Four Instillations with 87.5 mg of $\text{CaNa}_3\text{-DTPA}$ [An area of fibroblastic activity is present at (A). Alveolar septa appear more thickened than in Figure 2, and macrophage proliferation is more apparent. 200X]

FIGURE 4. Lung of Rat which Received Three Instillations with 100 mg of $\text{CaNa}_3\text{-DTPA}$ [Fibroblastic activity (A), bronchiolization of alveolar epithelium (B), and septal thickening due to macrophage proliferation and septal cell hyperplasia are present (arrows). 200x1]

These lesions consisted of a few focal areas of fibroplasia accompanied by lymphoid cell infiltration, a few scattered focal accumulations of macrophages, diffuse vesicular emphysema of varying severity, and scattered areas of atelectasis.

Lungs of rats which received ZnNa_3 -DTPA dosages of 50 to 87.5 mg showed only small scattered lesions of the type present in rats treated with comparable doses of CaNa_3 -DTPA.

Rats treated with ZnNa_3 -DTPA and permitted a 2-week recovery period before being euthanized showed minimal lesions similar in extent and severity to those present in CaNa_3 -DTPA subjects given a similar recovery period.

Rats allowed to recover 4 weeks after treatment with either CaNa_3 -DTPA or ZnNa_3 -DTPA did not show histologic alterations in their lungs.

Intratracheal treatment of rats with sodium citrate produced interstitial pneumonitis of a mild nature in two subjects and similar lesions compounded by patches of protein rich edema fluid flooded alveoli in another subject. Patchy areas of emphysema and atelectasis were also present; however, these were confined to quite small areas of the lung. Very mild or no lesions of consequence were present in sodium-citrate-treated rats euthanized 2 weeks and 4 weeks following treatment.

In sodium-chloride-treated rats, a few foci of atelectasis and emphysema were present along with some evidence of inflammation of a few bronchi and

adventitia of small vessels. No lesions of significance were present in sodium-chloride-treated animals which were permitted to recover for 2 and 4 weeks following treatment.

CONCLUSIONS

CaNa_3 -DTPA and ZnNa_3 -DTPA are lethal to rats when administered intratracheally in doses of 75 mg or greater. The maximum tolerated dose is on the order of 50 mg. In terms of a comparable dose in man, this would amount to 35 grams or approximately 35 times the recommended dosage for repeated therapeutic administration. Histopathologic changes induced by DTPA are those of a severe inflammatory response with alveolar cell metaplasia and hyperplasia leading to near total consolidation of the lung at the higher dosage levels. These lesions are repaired within 2 to 4 weeks after the drug is discontinued. The results indicate a greater acute toxicity of the intratracheally instilled drug compared to that following intraperitoneal injection (150 mg/rat/day for 16 days was tolerated). The dose employed in man appears to be well within reasonable safety limits for acute toxicity. Further studies are required to determine the chronic toxicity and pathology of inhaled DTPA as well as the combined effects of DTPA and Pu in the lung. The relative effectiveness of inhaled versus intravenous DTPA in removing Pu must also be considered.

A METHOD FOR INTRATRACHEAL INSTILLATION IN THE RAT

Investigator:

W. G. Morrow

A method was developed for the safe, rapid, and accurate intratracheal instillation of radionuclides into the rat lung. Rats anesthetized with halothane were suspended vertically on an animal support stand with rubber bands attached to the incisor teeth holding the mouth open. A speculum was inserted into the trachea and the radionuclide injected from a syringe through tubing inserted through the tracheal speculum into the trachea. Radionuclide lung burdens obtained by this method varied by less than a factor of two within groups of rats observed 4 hr to 20 days after instillation. Distribution of the radionuclide between the left and right side of the lung was similar to that obtained with inhalation when the instilled volume was 2 ml. Instillations of less than 2 ml showed unequal distribution of radionuclide between the right and left lung.

This method was developed to deposit ^{253}Es and ^{249}Bk in the lungs of rats because inhalation exposures were not possible due to the limited quantity of radioactive material. Simplicity, rapidity, safety, animal survival, and dose accuracy were the objectives in developing this technique. The method is a composite of published methods on intratracheal instillation in hamsters and new techniques which were needed to provide satisfactory results with rats because of anatomical and physiological differences.

OBSERVATIONS

Individual rats weighing 175-200 grams were anesthetized in a large desiccator, containing gauze sponges saturated with halothane, until eyelid movement ceased. The rat was

then removed from the desiccator and suspended vertically on an animal support stand by a rubber band passed under the upper incisor teeth. A second rubber band was then placed over the lower incisor teeth to hold the mouth open and the tongue was pulled to one side using a gauze to facilitate gripping (Figure 1). A speculum (made from an Argyle(R) umbilical artery catheter) was inserted into the trachea with the aid of a stiffener (made from a 4-inch, 20-gauge needle filed and bent to desired shape) (Figures 1 and 2). The speculum was moved slowly down to the epiglottis, then raised and lowered until the rat breathed, thereby opening the epiglottis and exposing the trachea. The tip of the speculum was felt rubbing down the cartilage of the trachea insuring proper implantation. The conical design of the speculum was chosen

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FIGURE 1. Anesthetized Rat **Suspended Vertically** on an Animal Support Stand with Rubber Bands (1) Under the Incisor Teeth, Holding the Mouth Open [The tracheal speculum (2) (Argyle (R) umbilical artery catheter) is being inserted with the speculum stiffener (3) while the tongue is pulled outward and to one side.]

so that its entry would be limited by the diameter of the trachea whereas it could readily pass all the way down the esophagus. The speculum stiffener was removed as soon as possible to allow unimpaired breathing. At this point, the rat was observed until he was very lightly anesthetized as determined by a response to speculum move-

ment similar to gagging. A 23-gauge, 3/4-inch needle and 2-1/2 cc syringe with a 2-inch piece of tubing (P.E. 50, I.D. 0.023 in. x O.D. 0.038 in.) attached was then used to administer the dose through the speculum. The speculum was left in place after instillation until the rat had almost revived. The speculum was then

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FIGURE 2. Materials Used for Intratracheal Instillation Included an Argyle (R) Umbilical Artery Catheter (1) (Right) Cut Off to Make a Trachea Speculum (2), a 20-Gauge Needle Filed and Bent to the Desired Shape (3) to Form a Stiffener for Inserting the Speculum into the Trachea, a Syringe with a 23-Gauge Disposable Needle Attached to a 2-Inch Piece of P.E. 50 Tubing (4), and Forceps (5) for Removing the Potentially Radio-nuclide Contaminated Speculum from the Rat.

removed from the trachea and the rat removed from the stand and placed in a cage for observation. Only minimal respiratory depression was observed while following this procedure and the animals were alert and active almost immediately.

Halothane was selected as the anesthetic after attempts to revive rats following instillation proved futile when using pentobarbital sodium, thi-amylal sodium, thiopental sodium, or ether. Halothane anesthetized the rat

rapidly and the average length of anesthesia was 1 to 3 min. This limited the rate at which animals could be instilled to an average of about one rat every 2 min.

Instillation of 0.5 cc could be followed within 1 hr by an additional 0.5 cc dosage with minimal respiratory depression. Saline lung lavage with repeated instillation and withdrawal of as much as 3.0 cc was well tolerated.

No radioactivity was detected on the nose or around the mouth of the rat following intratracheal instilla-

tion. After administering the dose, no "blow back" of the material through the speculum was observed, perhaps due to the depth at which the dose is instilled in the trachea and the upright position of the rat on the stand which appears to facilitate deposition and retention of the liquid.

A comparison of the behavior of inhaled and intratracheally instilled radionuclides was not the purpose of developing this technique; however, a difference in the range of lung burdens obtained in rats exposed by the two methods was observed (Table 1).

TABLE 1. Deposition of Inhaled Versus Intratracheally Instilled Radionuclides in Rats

<u>Inhalation</u> ^(a)	Time After Administration	<u>Lung Burden</u>	
		Mean	Range
¹⁴⁵ Sm ₂ O ₃	Immediately	3.9 nCi	1.4 - 8.9
¹⁴³ Pm ₂ O ₃	Immediately	4.8 nCi	0.4 - 2.4
²³⁸ PuO ₂	3 Days	6.8 nCi	4.5 - 9.6
¹⁴⁵ Sm ₂ O ₃	14 Days	2.4 nCi	0.7 - 7.7
²³⁹ PuO ₂	21 Days	44.2 nCi	10.3 - 98.4
<u>Intratracheal</u> ^(b)			
²⁵³ EsCl ₃	4 hr	1.29 µCi	1.06 - 1.45
²⁵³ EsCl ₃	1 Day	0.98 µCi	0.91 - 1.03
²³⁹ Pu(OH)	7 Days	0.19 µCi	0.17 - 0.22
²³⁹ Pu(NO ₃) ₄	7 Days	0.17 µCi	0.11 - 0.19
²⁵³ EsCl ₃	20 Days	0.18 µCi	0.14 - 0.22

a. Rats within each group were exposed to the same aerosol at the same time.

b. Rats within each group were instilled with identical volumes of the same radionuclide solution.

Less variability was seen in the radionuclide lung burdens of rats after intratracheal instillation than after inhalation. Radionuclide lung burdens obtained by intratracheal instillation varied by less than a factor of two within groups of rats observed 4 hr to 20 days after instillation.

The radionuclide was not equally distributed between the left and right lung with instilled volumes of less than 2.0 ml. This is probably inherent in the technique rather than due to any physiological variability. A more uniform distribution between the left and right lung was attained as the instilled volume was increased and at 2.0 ml was similar to that obtained with inhalation (Table 2).

CONCLUSION

When small quantities of radionuclides are available for study, intratracheal instillation is a convenient method of administering a lung burden to rats. The initial lung burden is accurately determined using this technique since all of the instilled nuclide is delivered to the lung. Lung burdens of rats intratracheally instilled showed much less variability than inhaled lung burdens

TABLE 2. Ratio of Radionuclide Between Left and Right Lungs

Administered Volume	Ratio ^(a)	
	Mean	Range
<u>Intratracheal</u>		
.5 cc	3.26	1.51 - 6.68
1.0 cc	2.57	1.54 - 3.59
1.5 cc	1.69	1.05 - 2.32
2.0 cc	1.10	1.09 - 1.12
<u>Inhalation</u>	1.08	1.06 - 1.10
<i>a. High lung/low lung radionuclide concentration ratio.</i>		

when measured after comparable periods of time.

Intubating the trachea is a simple technique and with a minimum of practice, a reasonable rate of instillation is achieved. Liquid volumes of 0.5 cc to 2.0 cc, including repeated saline lung washings of 3.0 cc each, were well tolerated by the rat. Since handling and instillation of the radionuclide is controlled at all times, hazards due to contamination of the rat and the work area are minimized.

DEVELOPMENT OF AN ISOLATED ORGAN PREPARATION FOR STUDYING
EFFECTS OF TOXIC MATERIALS ON LUNG TISSUE METABOLISM

Investigators:

J. G. Hadley and E. G. Tombropoulos

We are interested in the metabolic changes induced in lung tissue by various toxic materials in the lung. To further this interest, we have developed a system in which various biosynthetic processes occurring in rodent lung tissue can be studied while parameters associated with respiratory physiology, such as tidal and minute volumes, pulmonary arterial pressure, flow rate through the lungs, composition of inspired air, etc., are carefully controlled and can be varied independently of one another.

The preparation consists of the excised lung and trachea of a hamster, ventilated with pulses of negative pressure, while being perfused through the pulmonary vasculature. A simplified schematic drawing of this preparation is shown in Figure 1. The lung is housed in a glass container and suspended from the plexiglass lid through which pass the cannulae for the trachea and pulmonary artery. Pulses of negative pressure are exerted in the chamber causing the lung to inflate. Deflation is allowed by bleeding air back into the chamber between inflations. The trachea is attached to a humidified supply (at ambient pressure) of either room air or oxygen-enriched air. The perfusion media is drawn from a reservoir beneath the lung by a peristaltic pump and passed to the top of the H-shaped column. The media fills the column to the horizontal bar of the H. The height of this bar maintains the con-

stant perfusion pressure. Media in excess of that flowing through the lungs returns to the reservoir via the other leg of the H column. The perfusion media enters the lung through a cannula tied into the pulmonary artery. After coursing throughout the lung vasculature, it leaves the lung via the pulmonary veins and drops to the reservoir. The entire system is enclosed in a temperature controlled box. The operational parameters are given in Table 1.

Grossly, the perfused lung appeared normal, inflated easily, and was free of any signs of edema for at least 60 min. Histologically, no major structural changes were seen in the lung tissue following perfusion. Biochemically, the lung is known to be a very active organ in the synthesis of lipids from acetate and also to metabolize glucose with the subsequent release of lactic acid. Therefore, as an indicator of metabolic activity,

FIGURE 1. Schematic Diagram of Perfused Lung Preparation

TABLE 1. Operational Parameters for Hamster Lung Perfusion

Perfusate: Krebs Ringers phosphate buffered salt solution containing 6% bovine serum albumin and 1 mg/ml glucose, pH 7.3.

Perfusion Flow Rate: 2.0 - 2.5 ml/min

Perfusion Pressure: 15 cm H₂O

Respiration Rate: 70 ± 5 min

Tidal Volume: 1.0-1.5 ml

Temperature: 30 ± 2 °C

Time: Variable: Time periods from 10 min to 60 min have proved the most useful, allowing up to eight preparations to be completed by one operator in a single day.

we examined the utilization of acetate for long-chain fatty acid synthesis and also the lactic acid production by the perfused lung. Figures 2 and 3 show the results of these experiments. The linearity of both the curves throughout the 60-min time period indicated that the perfused lung is metabolically active during that period of investigation.

Aside from studies of the biosynthetic activity of the lung under various conditions, including following inhalation of radioactive particles, the lung perfusion preparation

should be useful in several other studies. The toxicity of various therapeutic agents administered by inhalation, e.g., DTPA, can be evaluated. The solubility and ultrafiltrability of variously sized particles, administered via the trachea, can be correlated with their rate of elimination via the bloodstream. The effect of therapeutic agents on this rate can also be studied.

FIGURE 2. ($1-^{14}\text{C}$) Acetate Incorporation by Perfused Hamster Lung (Each point average of three perfusions.)

FIGURE 3. Lactate Production (The perfusion medium was recycled and samples were taken for lactic acid determination by assaying for reduced β -DPNH from the lactic dehydrogenase reaction. Lactate + β -DPN $\rightarrow$ pyruvate + β -DPNH + H^+ . This represents 50 nanomoles/min per hamster lung.)

LOW-LEVEL PLUTONIUM INHALATION STUDIES IN BEAGLES

This project is concerned with a specific experiment to determine the long-term effects in beagle dogs of low-level inhalation exposure to plutonium dioxide. The experiment will extend previous and continuing studies in our laboratory on the effects of higher level exposures. This year's reports are concerned with the development and application of inhalation exposure apparatus and procedures. During the period from October 1970 to November 1971, 134 dogs were exposed to $^{239}\text{PuO}_2$ aerosols.

RELATIONSHIPS BETWEEN THE SIZE DISTRIBUTION OF $^{239}\text{PuO}_2$ AEROSOLS,
AEROSOL CONCENTRATION, AND NEBULIZER SUSPENSION CONCENTRATION

Investigators:

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Technical Assistance:

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In a study of low-level effects of inhaled $^{239}\text{PuO}_2$ in beagle dogs, alveolar burdens over the 1500-fold range from 2 nCi to 3 μCi were deposited in unanesthetized dogs by aerosol inhalation. Aerosols were generated by nebulizing magnetically stirred suspensions of $^{239}\text{PuO}_2$ particles in water; the aerosol concentration (CONC) being varied, primarily, by changing the Pu concentration of the suspension (SSA).

If the mass concentration of the suspension was kept below about 10 mg (540 μCi) PuO_2/mL , a good correlation ($R = 0.827$ for $n = 64$) was observed between CONC and SSA with constant air flow through the exposure chamber. SSA values from 1 to 600 $\mu\text{Ci}/\text{mL}$ gave CONC values in the range 1.5 to about 3000 nCi/L. However, the aerodynamic equivalent size distribution also varied significantly with CONC. The activity median aerodynamic diameter (AMAD) increased from about 1.5 to 3 μm as CONC increased ($R = 0.786$ for $n = 64$), while the geometric standard deviation (GSD) decreased ($R = 0.576$ versus AMAD).

These observations help to explain the large variation observed in the percentage alveolar deposition of inhaled $^{239}\text{PuO}_2$ aerosols from less than 1% to nearly 50% in 64 dogs for which the data are thus far available. A change in AMAD from 1.54 μm (GSD 2.61) to 2.77 μm (GSD 1.61) decreased the percentage of ^{239}Pu activity in particles having aerodynamic equivalent diameters less than 1 μm from 32.63 to 1.62. This effect on particle size is the major factor determining percentage alveolar deposition.

In a study of the low-level effects of inhaled $^{239}\text{PuO}_2$ in beagle dogs, alveolar burdens over the 1500-fold range from 2 nCi to 3 μCi were deposited in unanesthetized dogs by aerosol inhalation. Since these were acute rather than chronic exposure studies,

repeated exposures were not considered desirable as a method of achieving deposition over such a large range.

The time for which a dog can be subjected to nose-only exposure to a toxic aerosol is limited by practical considerations involving dog training time, the comfort of the dog and the animal-handling technicians, the length of time over which a constant

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concentration of a suitable aerosol can be generated, and the time required to obtain representative samples for characterization of the exposure conditions. The range applicable to the present experiment was from 2 to 40 minutes, a factor of 20. The volume of air inhaled by a dog was measured but is a variable over which we have no control. The 16- to 20-month-old beagles involved had minute volumes of between 1 and 5 l/min during their exposure.

The volume of air inhaled (l) times the aerosol concentration ($\mu\text{g/l}$ or $\mu\text{Ci/l}$) determines the quantity of material that is inhaled by the dog during its exposure. In the absence of other variables, it is this quantity that will determine how much of the material is deposited in the alveoli of the dog. Clearly, if it is desired to deposit alveolar burdens over a large range in a group of animals, the only parameter that can conveniently be changed by several orders of magnitude is the aerosol concentration. In the present experiment, the mean concentration of $^{239}\text{PuO}_2$ varied from 0.4 nCi/l to 3.86 $\mu\text{Ci/l}$.

It is reasonable to expect that the particle size distribution of the inhaled material will play an important part in determining its fractional deposition in various regions of the dog's respiratory tract. It therefore seemed important to investigate in detail the effect, if any, that changes in the aerosol mass concentration over nearly four orders of magnitude had on the particle size distribution of the $^{239}\text{PuO}_2$ aerosols to which the dogs were exposed.

METHODS

The exposure chambers, equipment, and techniques used for the dog inhalation experiments have been described elsewhere.^(1,2) plutonium-239 aerosols were generated by nebulizing magnetically stirred suspensions of particles in water with Lovelace nebulizers.⁽³⁾ The PuO_2 was prepared by calcining ^{239}Pu oxalate for 2 hr at 750 °C and making a suspension of mass concentration 20 mg/ml of triple distilled water. This contained many large particles which were not broken up by ultrasonic agitation. To eliminate large particles, the suspension was shaken vigorously by hand for 2 min and, after allowing it to stand for 10 min, the top 3.5 cm of supernatant was withdrawn. Spheres of diameter 3.34 μm and density 10.6 g/cm³ (Stokes' diameter for PuO_2) will settle this distance through water in 10 min. The Stokes' diameter distribution of particles in the supernatant was log-normal at the time of preparation with a mass median diameter (MMD) of 1.10 μm and a geometric standard deviation (GSD) of 1.96. This stock suspension had an activity concentration of about 100 $\mu\text{Ci/ml}$ and was either diluted or concentrated prior to nebulizing it, depending upon the dose group of the dogs being exposed. No dispersing agent was used in any of the suspensions because of the limited information available on the inhalation toxicity of these agents, but the containers were "dry filmed" with dimethyldichlorosilane to prevent Pu sticking to them.

The PuO_2 suspension to be used for a particular exposure was calibrated to determine its activity concentration (SSA); and, immediately prior to transferring it to the nebulizer bowl, it was ultrasonically agitated. A magnetic stirring bar was included in each suspension and the nebulizer was mounted on a magnetic stirrer during operation (Figure 1). Compressed air, usually at 20 p.s.i.g., was supplied to the nebulizer. Under these conditions, the output of the nebulizers used were in the range 45 to 100 μl liquid/min in approximately 1.6 l air/min under standard conditions, the exact flow depending upon the upstream air pressure and the diameter of the nebulizer jet. The output of the nebulizer was determined by dividing the difference in liquid volume before and after operation by the time of operation.

The nebulizer output was passed up a half-inch ID copper tube around which was wound a heating tape. Power was supplied to heat the air passing through the tube to between 125 and 150 $^{\circ}\text{C}$, the intention being to evaporate the water droplets containing the PuO_2 particles. The aerosol was mixed with diluting air at the top of the aerosol exposure chamber, the air being exhausted through two 47-mm diameter membrane filters at 10 l/min .

Aerosol concentration measurements were made by counting the activity on each of two 25 mm diameter membrane filters, through which air was drawn at a fixed rate for the duration of the dog exposure. Results from the two samples, expressed in nCi/ l , were

usually within a few percent of each other and their mean was taken as the chamber aerosol concentration. Point-to-plane electrostatic precipitator (ESP) ⁽⁴⁾ and Walkenhorst thermal precipitator (TP) ⁽⁵⁾ samples were taken under appropriate conditions to yield samples of the PuO_2 particles on electron microscope grids. These were shadowed with chromium at an angle of 26° and then examined in an electron microscope (EM). Figures 2 and 3 are micrographs of the PuO_2 aerosols, one being a low and the other a high concentration.

The difficulties involved in obtaining meaningful particle size distributions from EM samples have been discussed elsewhere. ⁽⁶⁾ samples for the aerodynamic equivalent size classification of the aerosols were taken with Mercer cascade impactors, ⁽⁷⁾ the sample duration being the same as the dog exposure. The filter papers and the silicone-coated glass slides from the impactor were sandwiched between two strips of cellophane adhesive tape and their activity determined by counting the ^{239}Pu 17 keV X rays under appropriate conditions. Mercer's effective cutoff diameter (ECD) method ⁽⁸⁾ was used in conjunction with a computer program to calculate the activity median aerodynamic diameter (AMAD) and the GSD of the PuO_2 aerosols, assumed to be log-normally distributed. (The aerodynamic equivalent diameter of a particle is the diameter of the unit density sphere which has the same final settling velocity as the particle. For a homogeneous material like PuO_2 , the

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FIGURE 1. Photograph of Nebulizer in Position on Magnetic Stirrer (Also shown is the coupling to the compressed air line and the heating tape wound around the aerosol inlet tube.)

AMAD and mass median aerodynamic diameter are the same and are related to the MMD by the square root of the particle density and a slip correction.)

The quantity of Pu inhaled by a dog was calculated by multiplying the

mean aerosol concentration in nCi/l (CONC) by the volume of air inspired by the dog.⁽¹⁾ The amount of alveolar deposited Pu was estimated by averaging the 14- and 28-day post-exposure, 17 keV X ray, thorax counts

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FIGURE 2. Electron Micrograph of $^{239}\text{PuO}_2$ Aerosol, Shadowed at 26° (TP sample, concentration $\sim 10\text{ nCi/l}$).

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FIGURE 3. Electron Micrograph of $^{239}\text{PuO}_2$ Aerosol, Shadowed at 26° (ESP sample, concentration $\sim 500\text{ nCi/l}$).

that were made for each dog.⁽⁹⁾ Plutonium cleared before this was assumed not to have been deposited in the alveoli.* This amount was divided by the quantity inhaled to obtain the percentage alveolar deposition (DEP) for each dog.

Some of the variables, measured as described above, during the exposure of 64 dogs, are given in Table 1. The CONC values have been ordered from lowest (0.42 nCi/l) to highest

(3862 nCi/l) for convenience. Linear correlation analyses were conducted between each relevant set of variables to seek significant relationships, the correlation coefficients (R) obtained being tested for significance at the $p < 0.05$ and the $p < 0.01$ levels. Where it appeared desirable, linear regression analyses were conducted to quantify the relationship between variables.

RESULTS

* Dogs sacrificed 14 days postexposure had 99% of their ^{239}Pu in the lung.

Some of the results obtained from the correlation analyses between

TABLE 1. Aerosol Size Distribution and Alveolar Deposition Data for Various Aerosol and Suspension Concentrations

Aerosol CONC nCi/l	Suspension Spec. Act. μCi/ml	Aerosol Size Distribution Data				Alveolar Deposition %	Aerosol CONC nCi/l	Suspension Spec. Act. μCi/ml	Aerosol Size Distribution Data				Alveolar Deposition %
		Parameters		Calculated % <1.0 μm	Parameters				Calculated % <1.0 μm				
		AMAD (μm)	G.S.D.		AMAD (μm)					G.S.D.			
0.42	0.767	2.11	2.48	20.55	100		196	81.8	2.31	1.77	7.13	10.3	
0.63	0.767	1.66	2.25	26.60	48.5 ^(a)		202	81.8	2.01	1.65	8.16	2.4	
1.00	0.767	1.81	1.88	17.36	2.6 ^(a)		220	81.8	2.12	1.76	9.19	7.8	
1.64	0.906	1.99	1.79	11.86	12.4 ^(a)		228	81.8	2.12	1.75	8.97	6.9	
1.69	0.906	2.01	1.86	13.03	0 ^(a)		229	495	2.60	1.66	2.97	3.7	
2.03	0.906	1.54	2.61	32.63	27.0		251	146	2.77	1.61	1.62	5.7	
2.30	0.908	1.89	2.03	18.43	16.2 ^(a)		271	107	2.43	1.78	6.18	3.5	
2.33	0.908	2.34	1.83	7.97	16.5 ^(a)		288	146	2.51	1.62	2.82	4.4	
2.54	0.767	1.66	2.27	26.82	28.3 ^(a)		317	495	2.60	1.60	2.10	4.1	
2.58	0.906	1.77	2.08	21.78	0 ^(a)		345	146	2.64	1.68	3.07	7.6	
3.13	0.906	1.65	2.23	26.62	15.5 ^(a)		388	102	2.11	1.80	10.20	6.8	
4.51	1.42	1.58	2.23	28.42	0 ^(a)		398	102	2.22	1.77	8.12	7.2	
4.85	1.42	2.08	2.46	20.79	0 ^(a)		504	102	2.18	1.74	7.97	5.5	
5.25	1.42	1.72	2.32	25.97	0 ^(a)		665	495	2.85	1.76	3.20	3.5	
5.30	4.45	2.13	1.76	9.05	13.3 ^(a)		689	643	2.76	1.80	4.21	3.4	
5.89	1.42	1.68	2.34	27.09	8.7 ^(a)		1142	478	2.43	1.78	6.18	3.5	
5.91	1.42	1.83	2.43	24.81	13.7		1143	415	2.76	1.71	2.92	2.6	
6.35	4.23	2.03	1.82	11.85	9.5		1156	478	2.43	1.72	5.08	3.8	
6.74	4.45	1.90	1.72	11.83	8.2 ^(a)		1293	415	2.79	1.68	2.40	1.8	
7.06	4.23	2.06	1.95	13.96	1.4 ^(a)		1310	415	2.63	1.73	3.89	3.6	
7.16	4.23	1.84	1.93	17.69	14.2		1907	643	2.78	1.78	3.81	4.6	
7.43	4.23	2.10	1.86	11.59	20.1		2121	489	2.91	1.83	3.86	2.6	
8.04	3.32	1.83	2.51	25.57	3.6		2257	489	2.75	1.83	4.71	1.4	
8.08	4.23	1.84	1.93	17.69	11.3		2432	489	2.97	1.78	2.95	2.2	
10.4	16.5	1.58	1.83	22.45	12.2		2477	614	2.81	1.73	2.97	7.9	
11.3	4.45	1.91	1.65	9.81	31.3		2520	643	2.74	1.81	4.47	5.4	
13.6	3.32	1.81	1.74	14.20	7.5		2569	489	2.86	1.79	3.55	2.1	
13.7	5.20	2.22	2.03	13.00	1.6		2592	489	3.07	1.78	2.59	2.6	
14.1	5.20	1.90	1.87	15.26	3.4		2690	489	2.84	1.84	4.35	1.8	
15.1	20.3	1.97	1.76	11.52	3.3		2725	614	2.93	1.77	2.99	10.5	
71.9	20.1	2.19	1.95	12.02	11.3		3759	489	2.85	1.76	3.20	2.2	
181	146	2.31	1.58	3.36	4.3		3862	489	2.80	1.77	3.57	3.6	

a. Thorax X ray count not significantly different from zero at $p < 0.05$.

variables are given in Table 2. The mean aerosol concentration in the chamber under fixed flow conditions (10 l/min) was significantly correlated with the nebulizer suspension specific activity ($R = 0.827$). CONC has been plotted against SSA on log $\times$ log paper in Figure 4. The correlation coefficient between $\log_{10}$ CONC and $\log_{10}$ SSA is 0.976, which is excellent considering the fact that the results presented were obtained during a series of dog exposures over a 5-month period. A number of different Lovelace nebulizers, having outputs ranging from 44 to 108 $\mu\text{l}/\text{min}$ with a mean of 76 $\mu\text{l}/\text{min}$, were involved. No adjustment to the CONC values was made on this account since only the mean output for each combination of

generator and suspension, involving from one to eight CONC values, was available. However, with only a couple of exceptions, points that fell far from the regression line in Figure 4 corresponded to nebulizer outputs that differed considerably from the mean value.

Both SSA and CONC are significantly correlated with the AMAD of the $^{239}\text{PuO}_2$ aerosol, the correlation coefficient between AMAD and CONC being 0.777. These variables have been plotted against each other in Figure 5 and a regression equation between AMAD and $\log_{10}$ CONC was computed. This gave a multiple correlation coefficient of 0.872, a highly significant value ($p \ll 0.01$). At the same time, GSD was observed to be negatively correlated with AMAD ($R = -0.576$) and, therefore, also with SSA and CONC. GSD has been plotted against AMAD in Figure 6, from which it appears that the above observation may be largely due to the few distributions having GSD values greater than 2.2. However, eliminating these values and recomputing the regression equation still gives coefficients that are significantly different from zero at the $p < 0.01$ level of significance.

The distribution parameters AMAD and GSD were calculated from the Mercer cascade impactor data, it being assumed that the activity was normally distributed with respect to the logarithm of the aerodynamic equivalent size. Since AMAD increased with CONC while GSD decreased, the fraction of activity on particles in various size ranges changed as the aerosol concentration changed.

TABLE 2. Correlations Between Some Measured Parameters

Variable		Linear Correlation Coefficient R	(N)	Significance Level ^(a)	
X	Y			p < 0.05	p < 0.01
SSA	CONC	0.827	(64)	Yes	Yes
SSA	AMAD	0.872	(64)	Yes	Yes
SSA	GSD	-0.443	(64)	Yes	Yes
CONC	AMAD	0.777	(64)	Yes	Yes
CONC	GSD	-0.310	(64)	Yes	No
AMAD	GSD	-0.576	(54)	Yes	Yes
DEP	S	-0.521	(52)	Yes	Yes
DEP	CONC	-0.423	(52)	Yes	Yes
DEP	AMAD	-0.625	(52)	Yes	Yes
DEP	GSD	0.461	(52)	Yes	Yes
DEP	CLS-1.0	0.642	(52)	Yes	Yes

(a) For N = 64, correlation coefficient is significant at $p < 0.05$ if $R > 0.246$ and at $p < 0.01$ if $R > 0.320$

For N = 52, correlation coefficient is significant at $p < 0.05$ if $R > 0.273$ and at $p < 0.01$ if $R > 0.354$

DEP - Percentage of inhaled material deposited in alveoli of dogs

SSA - Suspension specific activity in $\mu\text{Ci}/\text{ml}$

CONC - Aerosol concentration in nCi/l

AMAD - Activity median aerodynamic diameter (μm)

GSD - Geometric standard deviation

CLS-1.0 - Calculated fraction of activity on particles less than 1.0 μm diameter

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FIGURE 4. Graph of Mean $^{239}\text{PuO}_2$ Aerosol Concentration as a Function of Nebulizer Suspension Specific Activity

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FIGURE 5. Graph of Activity Median Aerodynamic Diameter as a Function of $^{239}\text{PuO}_2$ Concentration

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FIGURE 6. Geometric Standard Deviation as a Function of Activity Median Aerodynamic Diameter of $^{239}\text{PuO}_2$ Aerosols

The linear correlation coefficients between the percentage alveolar deposition (DEP) for each dog and a number of the variables of Table 1 are listed in Table 2. Ten values for which the alveolar burden was not significantly different from zero at $p < 0.05$ and two values for which the concentration was less than 1 nCi/l, too low to reliably determine the quantity of activity inhaled by those dogs, have been excluded. This gives $N = 52$ instead of $N = 64$. DEP is clearly well correlated with all the parameters listed.

DISCUSSION

The relationships observed between SSA, CONC, and the particle size distribution of the aerosol can be ex-

plained in terms of the probability of there being more than one PuO_2 particle per droplet escaping from the nebulizer. If we assume that the particles are uniformly distributed in the nebulizer suspension and that their concentration is directly proportional to SSA, this probability can be calculated if we know something about the distribution of droplet sizes escaping from the nebulizer. The results of some rough calculations along these lines are given in Table 3 for SSA values ranging from 1 $\mu\text{Ci/ml}$ to 1000 $\mu\text{Ci/ml}$.

Assuming that the Stokes' diameter distribution of particles in suspension remains the same as SSA changes, i.e., at $MMD = 1.10 \mu\text{m}$ and $GSD = 1.96$, the number of PuO_2 particles per unit volume increases from

TABLE 3. The Probability of Aggregate Formation in the Nebulization of Suspensions of Particles

Suspension Concentration (SSA) $\mu\text{Ci/ml}$	$^{239}\text{PuO}_2$ Particle Concentration $\#/ \text{ml}$	Droplet Emission Rate $\#/ \text{min}$	Droplets with Probability of Having More Than One Particle	Percentage Aggregates Formed %
1	2.2×10^7	5.9×10^9	1.5×10^4	2.5×10^{-4}
5	1.1×10^8	5.9×10^9	7.1×10^4	1.2×10^{-3}
10	2.2×10^8	5.9×10^9	3.4×10^5	5.7×10^{-3}
50	1.1×10^9	5.9×10^9	8.0×10^6	1.3×10^{-2}
100	2.2×10^9	5.9×10^9	2.8×10^7	4.7×10^{-1}
500	1.1×10^{10}	5.9×10^9	4.2×10^8	7.1
1000	2.2×10^{10}	5.9×10^9	1.1×10^9	19

2.2×10^7 to $2.2 \times 10^{10}/\text{ml}$ over the above SSA range. Mercer⁽³⁾ gives the approximate mass distribution of droplets escaping from the nebulizer as $\text{MMD} = 5.4 \mu\text{m}$, $\text{GSD} = 1.9$, from which we can calculate the distribution of droplets with respect to volume. The product of a droplet volume and the particle concentration yields the average number of particles that droplets of that size will contain.

Obviously, if this average is much less than unity, there is little chance that nebulizing a uniform suspension of particles will result in a size distribution that differs from that of the particles in the suspension. However, if we compute the relative number of droplets likely to have more than one particle, we will obtain an idea as to the number of aggregates there will be in the aerosol. The values given in Table 3 are

based on a nebulizer output of $76 \mu\text{l}/\text{min}$, the droplets being log-normally distributed with an MMD of $5.4 \mu\text{m}$ and a GSD of 1.9. Since the values are intended only to indicate order of magnitude effects, they have been rounded off to two significant figures and should not be taken too literally. A far more sophisticated analysis, taking into account both the particle and the droplet distributions and the droplets not escaping from the nebulizer, would be necessary to derive the expected aerosol size distribution as a function of SSA and CONC, which also depends upon the total air flow through the exposure chamber. The values in the last column of Table 3 provide an explanation of the differences between the aerosols shown in Figure 2 for a concentration of about $10 \text{ nCi}/\text{l}$ and in Figure 3 for a concentration of about

500 nCi/l. The reason for the decrease in GSD as AMAD and CONC increase is believed to be that, since small particles are relatively more numerous than large ones (for MMD = 1.1 μm and GSD = 2.0, the CMD = 0.26 μm), they are more likely to be involved in aggregation. Aggregates of large particles are also heavier and more likely to be lost by sedimentation (in the horizontal section of the intake line) and impaction (in the mixer). The combined effect of these mechanisms is to decrease the range of particle sizes, hence the GSD of the distribution.

The fact that pulmonary deposition of aerosols is a function of the particle size distribution of the inhaled aerosol is, of course, well established. However, the published curves relating percentage deposition to particle size (for man)⁽¹⁰⁾ span three orders of magnitude in particle diameters. Under fixed respiration conditions, the percentage deposition changes by less than a factor of two as the particle diameter increases from 0.4 μm to 4 μm . For the 52 dogs included in the above analyses, DEP varied from 1.4 to 31.2%, while the size distribution parameters AMAD and GSD varied from 1.54 to 3.07 μm , and 1.58 to 2.61, respectively. The effect that these variations have on the percentage of the $^{239}\text{PuO}_2$ on particles of aerodynamic equivalent diameter less than 1 μm can be seen in Table 1, viz. 1.62% to 32.63% for calculated values. They lead to a correlation between DEP and CLS-1.0 of 0.642, which is significant at the $p < 0.01$ level.

CONCLUSIONS

A highly significant correlation has been shown ($p \ll 0.01$) between the concentration of Pu in magnetically stirred suspensions (SSA) and the aerosol concentration (CONC) obtained by nebulizing the suspensions under constant conditions. However, significant correlations ($p < 0.01$) have also been obtained between both SSA and CONC and the aerodynamic equivalent size distribution of the aerosol. An explanation of this phenomenon based on aggregate formation due to the greater likelihood of there being more than one PuO_2 particle per nebulizer droplet at the higher SSA values has been advanced.

The importance of the relationship between CONC and the aerosol size distribution lies in the effect of the latter on DEP. The correlation between the percentage alveolar deposition and the percentage of $^{239}\text{PuO}_2$ on aerodynamic equivalent diameters less than 1 μm (CLS-1.0) is significant at the $p < 0.01$ level. For 52 dogs, DEP varied from 1.4 to 31.3% and CLS-1.0 varied from 1.62 to 32.62%. Although this excludes 12 dogs for which DEP could not be accurately determined, their inclusion would not have altered the above conclusions.

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ALVEOLAR DEPOSITION OF $^{239}\text{PuO}_2$ AEROSOLS IN BEAGLE DOGS
AS A FUNCTION OF RESPIRATION AND AEROSOL PARAMETERS

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Studies to determine the biological effects and metabolism of inhaled $^{239}\text{PuO}_2$ and $^{238}\text{PuO}_2$ in beagle dogs require predetermined amounts of plutonium to be deposited in the dogs assigned to each of six dose levels ranging from 2 nCi to 3 μCi . Alveolar deposition was estimated from 17 keV X ray thorax counts 14 days postexposure. Since percentage alveolar deposition varied from 0-54 during exposure of the first 36 dogs, a correlation matrix involving aerosol, respiration, and physiologic parameters was computed to identify those factors which most significantly influenced alveolar deposition. Particle size distribution and tidal volume accounted for 74% of the variability in deposition. Unlike tidal volume, respiration rate was not significantly correlated with alveolar deposition. Activity median aerodynamic diameter (AMAD) of the aerosol was found to be highly correlated with aerosol concentration (CONC), while the geometric standard deviation was significantly correlated with AMAD. This suggested that the pre-exposure value of CONC could be used indirectly to compute the percentage activity less than 0.5 μm aerodynamic equivalent diameter, the fraction of the aerosol most likely to be deposited in the alveoli. Since an estimate of tidal volume and aerosol concentration could be obtained during exposure, alveolar deposition values could be estimated from the suggested regression equation, and the volume that a dog should inhale to obtain the required alveolar burden could be calculated. Using these equations with a programmed computer during exposure, the desired alveolar burden was controlled to within a factor of about three during exposure of 31 dogs.

Studies to determine the biological effects and metabolism of inhaled $^{239}\text{PuO}_2$ and $^{238}\text{PuO}_2$ in beagle dogs require predetermined amounts of Pu to be deposited in the dogs assigned to each of six dose levels ranging from 2 nCi to 3 μCi . Since the percentage of alveolar deposition was found to

vary by orders of magnitude from dog to dog, experiments were initiated to determine the parameters most responsible for this variation and to use the results to control deposition of the desired alveolar burden in the dogs.

METHODS

Beagle dogs of both sexes were exposed to aerosols generated by nebulizing magnetically stirred suspensions of $^{239}\text{PuO}_2$ particles in water. The desired alveolar burdens range from 2 nCi to 3 μCi per 10 kg dog, divided into six exposure groups. The aerosol exposures were characterized with several monitoring devices. These included a venturi tidal volume air monitor (TVAM) through which the dog inhales the aerosol, and a remotely controlled plutonium aerosol concentration monitor (PAM), both of which are described elsewhere in this Annual Report. Since both aerosol concentration (CONC) and the volume of air inspired per unit time were measured during the exposure, we were able to calculate the amount of activity being inhaled by each dog. This information was used to estimate inspiration volumes (VOL) for inhalation of a preselected quantity of Pu, based on an assumed percentage alveolar deposition, for each of 36 dogs.

The PuO_2 was prepared by calcining ^{239}Pu oxalate for 2 hr at 750 °C and was suspended in triple-distilled water at a concentration of 20 mg PuO_2/ml . This suspension was shaken vigorously by hand for 2 min. It was then allowed to stand long enough for equivalent spheres of diameter larger than about 3.3 μm to settle out, after which the supernatant was withdrawn. (Spheres of diameter 3.34 μm and density 10.6 g/cm³ have a sedimentation velocity in water of 3.5 mm/min.) This stock suspension had a concentration of about 100 $\mu\text{Ci}/\text{ml}$ and was ei-

ther diluted with water or concentrated by controlled evaporation of the water, depending upon the dose group of the dogs being exposed.

Coincident with each dog exposure, two membrane filter paper samples (Millipore HA or Gelman GA6) and a Mercer cascade impactor sample were drawn from the aerosol chamber under calibrated conditions. Activity on filter papers and the silicone-coated glass slides from the impactor was determined by counting the ^{239}Pu 17 keV X rays. Aerodynamic equivalent particle size distributions [parameters: activity median aerodynamic diameter (AMAD) and geometric standard deviation (GSD)] were determined for each aerosol from the radioactivity measured on the seven impactor stages and the backup filter, using Mercer's effective cut-off diameter method.

The alveolar-deposited $^{239}\text{PuO}_2$ was determined from 17 keV X ray thorax counts 14 days postexposure. All dogs were counted for 30 min prior to exposure, the result being subtracted from the postexposure count to yield the net count. Analysis of the distribution of Pu in the tissues of eight dogs that were sacrificed 7 to 14 days postexposure showed that more than 99 percent was in the lung. We ignored alveolar-deposited material that was cleared within 14 days. The percentage alveolar deposition (DEP) is defined for the present purposes by the formula:

$$\text{DEP} = \frac{\text{NCI}}{\text{VOL} \times \text{CONC}} \times 100 \text{-----} \quad (1)$$

where:

NCI = nCi activity deposited in alveolar regions determined from the thorax counts
 VOL = volume of air inhaled by the dog in liters (ℓ)
 CONC = aerosol concentration in nCi/ℓ.

Tidal volume data, available in the form of a print-out of the volume of each inspiration and the total volume inhaled during a measured time interval, were used to determine the mean tidal volume (TV) and the mean respiration rate (RR). The product of these two parameters yields the mean minute volume of the dog (MV). Other parameters considered were the age in months, the weight (WT) in kg, and the sex of each dog.

Using the three aerosol parameters defined above (AMAD, GSD, and CONC) and the six respiration and/or dog parameters (TV, RR, MV, VOL, WT, and DEP), a simple correlation matrix was calculated in an attempt to determine those factors that most markedly vary with DEP and to investigate any other suggested relationships. Since both AMAD and GSD proved to be significantly correlated with DEP ($P < 0.01$), these two parameters were arbitrarily combined into one distributional descriptor, the percentage activity in particles having an aerodynamic equivalent size less than $0.5 \mu\text{m}$ (LS 0.5). We felt this avoided possible spurious correlations and gave a more precise estimate of particles with a high probability of deep lung penetration.

The above data were then used to conduct a stepwise linear regression, taking $Y = \text{DEP}$ and utilizing all the relevant measured parameters. This procedure tentatively identified the two parameters that explained a high

percentage of the observed variability in DEP. Attention was then focused upon these two parameters to obtain a relationship between them and DEP which could be used to enable us to more accurately achieve the desired doses in the dogs. Following the above data analysis, a further group of 31 dogs was exposed in the same manner as the first group, except that the results of the analysis were programmed into the computer in such a way that CONC and TV values, available during the exposure, could be used to predict the necessary exposure duration.

RESULTS

All pertinent data for the aerosols, dog respiration, and other physiologic measures, and deposition calculations are summarized in Table 1. The correlations between the parameters of interest are summarized in Table 2 together with their means, standard deviations, and significance of their correlations at the 5 and 1% levels.

Two deposition values in Table 1 were adjusted from 0 to 0.01% because the statistical variability in the count rates involved made it impossible (at the 95% confidence level) to distinguish an alveolar burden of less than 10 nCi from zero. This was done to prevent the computer from giving erroneous results. The thorax counter yielded 0.5 cpm/nCi ^{239}Pu in a dog-lung phantom; the background was of the order of 20 cpm; dog backgrounds were between 8 and 14 cpm; all counts were taken over a 30-min time period.

TABLE 1. Aerosol and Respiration Data for 36 Dogs(a)

Dog #	Aerosol Data				Respiration and Dog Data						Deposition	
	AMAD μm	CSD	CONC nCi/l	LS 0.5 %	TV ml/min	RR ₁ min ⁻¹	MV l/min	VOL l	WT kg	AGE mo	ACI nCi	DEP %
825F	2.26	1.93	2.33	1.3	137	13.0	1.78	10.4	10.9	17.6	8	32.9
841F	1.80	2.19	2.30	5.3	133	16.7	2.22	16.1	7.1	17.2	12	32.4
831F	1.85	1.70	11.3	0.9	341	13.3	4.53	6.8	9.4	17.4	28	36.5
845F	1.81	1.79	6.74	1.6	229	12.6	2.08	34.6	12.3	17.1	16	6.9
862M	2.04	1.84	5.30	1.2	225	19.1	4.30	19.8	12.2	16.7	12	11.4
834F	2.56	1.73	344.9	0.20	164	22.7	3.73	2.8	9.1	17.3	76	7.9
838M	2.22	1.62	180.9	0.13	139	15.5	2.15	7.9	10.6	17.3	70	4.9
844F	2.42	1.66	288.4	0.13	169	12.4	2.10	11.0	10.4	17.1	134	4.2
860M	2.68	1.65	251.4	0.05	175	20.6	3.60	18.9	13.0	16.7	270	5.7
820F	2.77	1.81	664.5	0.24	123	16.5	2.03	38.6	7.9	17.7	936	3.6
824F	2.51	1.64	316.7	0.06	193	15.3	2.96	20.1	10.8	17.6	272	4.3
819F	2.51	1.70	228.6	0.15	150	14.1	2.11	20.8	8.2	17.7	184	3.9
805F	3.11	1.73	394.7	0.046	115	19.9	2.28	19.4	7.7	17.9	280	3.7
857M	3.08	1.76	301.0	0.085	265	14.2	3.76	47.0	12.3	16.7	548	3.9
836M	2.72	1.64	259.7	0.033	172	13.4	2.30	53.9	13.9	17.3	288	2.1
840F	2.72	1.76	567.2	0.15	205	12.7	2.60	41.2	10.6	17.2	842	3.6
847M	1.90	1.87	1.64	1.65	267	8.2	2.20	14.7	10.9	18.4	6	24.9
853M	1.92	1.94	1.69	2.2	141	29.3	4.13	9.3	9.9	18.1	0.002(b)	0.01(b)
865F	1.68	2.23	2.58	7.0	166	16.3	2.70	11.6	9.5	17.3	0.003(b)	0.01(b)
870F	1.55	2.42	3.13	10.4	184	18.1	3.32	10.3	11.6	16.8	10	31.1
875F	1.47	2.98	2.03	16.5	364	12.2	4.43	16.4	14.0	16.7	18	54.1
826F	2.02	1.96	7.43	2.1	227	12.3	2.80	13.4	9.1	19.0	22	22.1
842M	1.93	1.90	6.35	1.7	213	15.9	3.39	21.5	11.1	18.5	14	10.3
867M	1.98	2.08	7.06	3.1	168	16.1	2.71	19.5	12.0	17.3	4	2.9
871M	1.75	2.04	8.08	4.0	222	12.7	2.82	14.2	11.9	16.8	8	7.0
874M	1.74	2.05	7.16	4.0	187	16.0	2.99	16.8	11.3	16.7	18	15.0
859M	1.89	1.84	15.1	1.4	201	14.0	2.83	100.1	14.2	18.1	50	3.3
866M	2.35	1.84	270.8	0.62	95	34.3	3.28	22.5	11.3	17.3	218	3.6
863F	2.89	1.84	2432	0.25	128	13.9	1.77	19.3	10.4	17.3	1860	4.0
804M	2.67	1.90	2257	0.50	219	13.3	2.92	44.5	12.3	20.4	1306	1.3
817M	2.82	1.90	2121	0.42	152	18.4	2.79	61.0	12.4	19.1	3478	2.7
829M	2.99	1.84	2592	0.17	198	14.6	2.88	57.6	13.6	19.0	4190	2.8
837M	2.78	1.85	2569	0.31	199	12.8	2.56	25.3	13.4	18.7	1412	2.2
856F	2.76	1.82	3759	0.08	173	17.6	3.06	10.4	11.6	18.1	890	2.3
864F	2.76	1.92	2690	0.50	249	9.9	2.46	17.7	10.3	17.3	892	1.9
873M	2.72	1.83	3862	0.28	282	8.7	2.51	13.3	13.6	16.7	1884	3.7
Means	2.32	1.89	730	1.91	194	15.7	2.89	24.7	11.1	17.5	360	10.2

a. Abbreviations defined in text.

b. Adjusted from 0. (See Text)

Table 2 shows that there is a significant correlation between DEP and both AMAD and GSD. The single descriptor related to the aerosol aerodynamic size distribution, LS 0.5, is also significantly correlated with DEP ($R = 0.675$). GSD is very highly correlated with LS 0.5 ($R = 0.955$) because the geometric standard deviation of the distribution is directly proportional to the "spread" of the distribution. An expected correlation between AMAD and CONC occurs since the higher aerosol concentrations were

achieved by nebulizing suspensions having higher plutonium concentrations, thereby increasing the probability of there being more than one PuO_2 particle per droplet. Of the physiological parameters investigated, only tidal volume is significantly correlated with the percentage alveolar deposition ($R = 0.470$).

Stepwise linear regression analyses were performed using these data, setting $\text{DEP} = Y$. This procedure identified $X_1 = \text{LS } 0.5$ as the parameter that permitted explanation of the largest

TABLE 2. Correlations Between Some Measured Parameters

Variable		Correlation Coefficient	Significance Level(a)	
X	Y		p < 0.05	p < 0.01
AMAD	GSD	-0.597	Yes	Yes
AMAD	Conc.	0.611	Yes	Yes
AMAD	LS 0.5	-0.690	Yes	Yes
GSD	Conc.	-0.144	No	No
GSD	LS 0.5	0.955	Yes	Yes
TV	RR	-0.564	Yes	Yes
TV	WT	0.397	Yes	No
RR	WT	-0.194	No	No
MV	WT	0.250	No	No
DEP	AMAD	-0.587	Yes	Yes
DEP	GSD	0.626	Yes	Yes
DEP	Conc.	-0.370	Yes	No
DEP	LS 0.5	0.675	Yes	Yes
DEP	TV	0.470	Yes	Yes
DEP	RR	-0.194	No	No
DEP	WT	-0.065	No	No

a. For $N = 36$, correlation coefficient is significant at $P < 0.05$ if $R > 0.325$, and at $P < 0.01$ if $R > 0.418$.

amount of residual variability, followed by $X_2 = TV$. Models involving these two parameters both with and without an intercept were tried, viz. $Y = a + b_1 X_1 + b_2 X_2$ and $Y = b_1 X_1 + b_2 X_2$. In each case, the model with no intercept allowed the better fit to the observed Y values, which were weighted by the factor $\sqrt{NCI}/100$ (chosen since it is mathematically related to the precision with which Pu activity in the lung can be determined). Various modifications were tried, such as excluding all values for which $NCI < 10$ nCi and $NCI < 30$ nCi, etc. The results of some

of these trials are summarized in Table 3. On the basis of the statistical evaluation, the equation that included all 36 data points and gave the best fit to the observed Y values was:

$$Y = 3.11 X_1 + 0.015 X_2 \text{-----} (2)$$

The multiple correlation for this equation (Table 3) is $R = 0.860$, and 74.0% ($0.860^2 \times 100$) of the observed variability in Y was explained.

The coefficients b_1 and b_2 obtained when data points involving NCI values less than 10 nCi were excluded are for

TABLE 3. Linear Regression Models Involving Two Independent Variables

Input Data		Regression Equation $Y = B_1X_1 + B_2X_2$								Statistical Evaluation		
# Pts.	Points Included	B_1	S.D. (b)	Sig. Level, P <	X_1		S.D. (b)	Sig. Level, P <	X_2	Von Neuman Ratio (e)	Resid. Std. Dev. (f)	Mult. Corr. Coef.
36	All (a)	3.11	0.46	0.001	LS 0.5	0.015	0.004	0.001	TV	1.08	1.82	0.860
36	All (a)	2.99	0.46	0.001	LS 0.5	0.020	0.005	0.001	ETV ₁ ^(c)	0.95	1.84	0.853
36	All (a)	3.11	0.45	0.001	LS 0.5	0.024	0.006	0.001	ETV ₂ ^(d)	1.09	1.80	0.859
34	DEP > 0	3.12	0.46	0.001	LS 0.5	0.015	0.004	0.001	TV	1.03	1.85	0.854
29	NCI > 10	3.25	0.49	0.001	LS 0.5	0.015	0.004	0.001	TV	1.11	1.74	0.878
21	NCI > 30	-0.325	1.716	NS	LS 0.5	0.016	0.003	0.001	TV	1.64	0.85	0.894

a. Zero values adjusted to 0.01%

b. S.D. = Standard deviation of corresponding coefficient

c. $ETV_1 = TV - ADS_1 = TV - 2.2 WT$

where: ETV = effective tidal volume (ml)

TV = measured mean tidal volume (ml)

ADS₁ = anatomical dead space, assumed

= $2.2 \times \text{Dog weight in kg}$

d. $ETV_2 = TV - TDS = TV - (ADS_2 + EDS) = 0.75 TV - 25$

where: TDS = total dead space

ADS₂ = anatomical dead space, assume = 0.25 TV, and

EDS = external dead space in venturi and mask = 25 ml

e. Von Neuman's ratio measures the randomness of the scatter of points about the fitted line, low values indicating a trend in the data.

f. Residual Std. Dev. should be as low as possible for a good fit of equation to points.

all practical purposes the same as those for Equation 2. Deposited Pu values greater than 10 nCi can be determined in the whole-body counter with increasing confidence, giving more credence to the DEP values calculated from Equation 1. However, elimination of all values involving NCI < 30 nCi altered the nature of the calculated equation. For the 21 remaining data points, the equation obtained for weighted values was:

$$Y = (-0.325 \pm 1.716) X_1 + (0.016 \pm 0.003) X_2 \text{-----(3)}$$

The coefficient of X_1 is not significantly different from zero and the

percentage alveolar deposition depended mainly on the mean tidal volume of the dogs during exposures. The mean value of the parameter LS 0.5 is 1.91 for all 36 points, 4.2 for the 15 points for which NCI < 30 nCi, and 0.27 for the remaining 21 points. Elimination of the low NCI values also eliminates all the high percentage alveolar deposition values [$\bar{Y}(21) = 3.20 \pm 0.6\%$ compared with $\bar{Y}(36) = 10.2 \pm 12.8\%$]. The relationship between particle size and percent alveolar deposition is obvious.

Since not all the air inspired will reach the pulmonary region, the amount of aerosol available for deposition is somewhat less than that contained in

the mean tidal volume. An effective tidal volume (ETV_1 or ETV_2), corrected for dead space, was therefore employed in some of the models summarized in Table 3. Little direct information on the anatomical dead space (ADS) of the beagle dog is available. Arbitrarily, we assumed that lung weight was proportional to dog weight and that ADS was similarly related. This approach led to ETV_1 , as defined in Table 4. Dr. J. L. Mauderly at the Lovelace Founda-

tion (personal communication) found the anatomical dead space to be a fairly constant fraction of the mean tidal volume of the unanesthetized beagle dog which led us to compute ETV_2 . As can be seen from the coefficients and statistics presented in Table 3, these adjustments to the tidal volume made only marginal differences in the fitted equations and did not alter our conclusions.

TABLE 4. Comparison Between Observed and Predicted X_1 and Y Values

DOG #	CNC from P.A.M. nCi/l	Calculated		X = % LS 0.5			X_2 TV (ml)	Y = % Alveolar Deposition		
		AMAD (μ m)	GSD	Observed	Calculated	Ratio		Observed	Predicted	Ratio
849	0.46	1.53	2.15	5.65	7.16	0.19	154	100	24.6	4.07
850	0.46	1.53	2.15	6.95	7.16	0.91	151	48.5	24.6	1.97
879	0.40	1.51	2.15	2.08	7.51	0.28	151	2.6	25.6	0.10
893	1.03	1.66	2.11	7.16	5.41	1.32	239	28.3	20.5	1.38
853	5.65	1.92	2.02	7.93	2.82	2.81	258	3.6	12.6	0.29
877	2.72	1.81	2.06	1.01	3.77	0.27	95.6	7.5	13.2	0.57
881	10.3	2.01	1.99	2.85	2.18	1.31	137	12.2	8.8	1.39
848	237	2.50	1.84	0.37	0.41	0.90	113	10.3	3.9	2.64
851	219	2.49	1.84	0.49	0.44	1.11	142	6.9	3.5	1.97
876	208	2.48	1.85	0.27	0.45	0.60	87.5	2.4	2.7	0.89
883	143	2.42	1.86	0.53	0.57	0.93	354	7.8	7.1	1.10
884	1696	2.80	1.74	0.05	0.09	0.56	148	1.8	2.5	0.72
888	1339	2.77	1.75	0.12	0.12	1.00	222	3.6	3.7	0.97
854	3024	2.89	1.71	0.07	0.06	1.17	169	2.6	2.7	0.96
852	3304	2.91	1.71	0.18	0.05	3.60	185	3.8	2.9	1.31
880	3636	2.92	1.70	0.31	0.05	6.20	152	3.5	2.4	1.46
891	6.74	1.95	2.01	7.20	2.62	2.75	284	13.7	12.4	1.10
900	6.89	1.95	2.01	7.70	2.59	2.97	156	8.7	10.4	0.84
904	5.45	1.91	2.03	7.10	2.86	2.48	93.1	0.01 ^(a)	10.3	----
907	4.65	1.89	2.03	7.57	3.05	2.48	75.7	0.01 ^(a)	10.6	----
899	15.4	2.07	1.97	1.76	1.82	0.97	75.5	1.6	6.8	0.24
908	16.4	2.08	1.97	1.65	1.77	0.93	105	3.4	7.1	0.48
905	79.1	2.33	1.89	1.35	0.80	1.69	82.8	11.3	3.7	3.05
892	638	2.65	1.79	0.45	0.21	2.14	218	7.2	3.9	1.85
898	233	2.50	1.84	0.72	0.42	1.72	137	6.8	3.1	2.00
909	694	2.66	1.79	0.39	0.20	1.95	130	5.5	2.6	2.12
889	347	2.56	1.82	0.18	0.32	0.56	104	3.4	2.6	1.31
890	1693	2.80	1.74	0.15	0.09	1.67	129	4.6	2.2	2.09
896	1576	2.79	1.75	0.21	0.10	2.10	105	5.4	1.9	2.84
906	1780	2.81	1.74	0.08	0.09	0.89	173	7.9	2.9	2.72
910	2791	2.88	1.72	0.10	0.06	1.67	155	10.5	2.5	4.2
MEAN								10.8	7.9	

Number of Ratios within Range 0.5 to 2.0

for X_1 , 20 = 64.5%

for Y, 17 = 54.8%

Number of Ratios within Range 0.33 to 3.0

for X_1 , 27 = 87.1%

for Y, 23 = 74.2%

P.A.M. = Plutonium Air Monitor

a. Values adjusted from zero

DISCUSSION

The results presented show that it is possible to describe the variability in the observed percentage of inspired aerosol deposited in the alveolar regions of the beagle lung (DEP = Y) in terms of just two independent variables. These are $X_1 = \text{LS } 0.5$, which described the aerodynamic particle size distribution in terms of a single parameter that might logically be expected to be related to alveolar deposition, and $X_2 = \text{TV}$, the mean volume of air inspired per breath by the dog during exposure. Together these parameters yield an equation (2 above) having a multiple correlation of 0.860, which accounts for 74% of the variability.

We considered Equation 2 adequate for a starting point to attempt to predict Y in future exposures. X_2 , the mean tidal volume, is measured during the course of the exposure; it is the total volume inhaled by the dog divided by the number of breaths to that time. X_1 is not so easily obtained but can be estimated from CONC which is measured immediately prior to the commencement of each dog exposure. Table 2 shows that the correlation of 0.611 between AMAD and CONC is significant at the 1% level. The large range of CONC values (1.64 nCi/l to 3.862 $\mu\text{Ci/l}$) suggested fitting a regression equation to AMAD and the logarithm of the concentration. This gave:

$$\text{AMAD} = (1.650 \pm 0.068) + (0.357 \pm 0.031) \text{LOG}_{10} \text{CONC} \text{---(4)}$$

with $R = 0.895$ and $\text{RSQ} = 0.800$, considerably higher than the linear correlation. The observed data and the fitted line have been plotted in Figure 1.

Table 2 also gives the correlation between GSD and AMAD, $R = -0.597$ ($P < 0.01$). The linear regression equation relating these two parameters is:

$$\text{GSD} = (2.63 \pm 0.17) - (0.312 \pm 0.073) \text{AMAD} \text{---(5)}$$

with both coefficients significantly different from zero ($P < 0.001$). These data have been plotted in Figure 2. Using these calculated AMAD and GSD values, the percentage of the aerosol below $0.5 \mu\text{m}$ can be calculated from the equation:

$$\text{CALC LS } 0.5 = 100 \int_{-\infty}^Z \frac{1}{\sqrt{2\pi}} e^{-\frac{Z^2}{2}} dZ \text{---(6)}$$

$$\text{where } Z = \frac{\ln 0.5 - \ln (\text{CALC AMAD})}{\ln (\text{CALC GSD})} \text{---(7)}.$$

We now have a value, $X_1 = \text{CALC LS } 0.5$, for insertion into Equation 3 to yield an estimate of Y(=DEP) based upon the measured CONC and TV.

The volume of air a dog is required to inhale from the aerosol chamber in order to deposit the desired dose in the pulmonary regions of the lung can now be calculated from Equation 1. Initially, Equations 1, 2, and 4 through 7 were programmed into the computer. NCI and CONC were typed into the computer terminal at the start of the exposure. The total

Neg 714288-2

FIGURE 1. Aerodynamic Median Activity Diameter (μm) Versus Logarithm of Aerosol Concentration (nCi/l) (with 95% confidence limits of regression line)

Neg 714288-1

FIGURE 2. Geometric Standard Deviation of Aerosol Distribution Versus Aerodynamic Median Activity Diameter (μm) (with 95% confidence limits of regression line)

volume and the number of respirations were typed in at convenient intervals to obtain an updated value for VOL as the exposure progressed.

The results obtained by following the above procedure in exposing the second group of dogs are presented in Table 4. To determine the adequacy of the predictive Equations 4, 5, and 2, the ratio of the observed to the calculated X_1 values, and observed to predicted Y values, were computed and are included in Table 4. Inspection of the last column shows that, aside from a few of the results at very low aerosol concentration, the predicted percentage alveolar deposition seldom differed from the observed value by more than a factor of three, five of eight values outside this range being for aerosol concentrations of less than 6 nCi/l. Fifty-five percent of the Y values were within a factor of two and 74% within a factor of three of the observed values. By contrast, for the first group of 36 dogs, only 33% of the deposited doses were within a factor of two and 56% within a factor of three of the desired doses.

Some improvements can be made in the above procedure. The Pu aerosol concentration monitor will be modified to measure both total concentration (CONC) and the concentration of activity on particles smaller than any desired cut-off size, such as CONC LS 0.5, by drawing the sample through an appropriate size selection device. Only mean values of the respiration parameters TV and RR were used in the above analyses, ignoring a large and

variable scatter in the data, which probably explains why the attempts to use an effective tidal volume (see Table 3) failed to improve the multiple correlations. Theoretically, the percentage alveolar deposition could be computed for each breath and the quantity estimated to have been deposited could then be integrated by an on-line computer until the desired NCI was reached.

CONCLUSION

The above analysis has shown that, while the percentage of inhaled particles that is deposited in pulmonary tissues can vary by orders of magnitude as a result of seemingly small changes in the important parameters, we can predict the percentage within a factor of about three. For the beagle dog inhaling $^{239}\text{PuO}_2$, the important parameters have been shown to be the aerodynamic particle size distribution of the aerosol and the tidal volume of the dog during exposure. For humans accidentally exposed to radioactive aerosols, these parameters are usually not known, but must be estimated from available data (aerosol concentration measurements, assumed work rate of the individual, etc.) or measurements made subsequently in an experimental simulation of the accident. It is hardly surprising that the results of computations based upon such measurements frequently differ by orders of magnitude from measured body burdens.

RELATIONSHIPS BETWEEN RESPIRATION PARAMETERS
OF UNANESTHETIZED ADULT BEAGLE DOGS DURING
TRAINING AND DURING $^{239}\text{PuO}_2$ AEROSOL INHALATION EXPOSURES

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Prior to subjection of dogs to aerosol inhalation exposures, they are trained daily for 2 weeks to familiarize them with the exposure procedures. Respiration rate and volume are measured and used to estimate the volume of air inhaled by each dog during its exposure to aerosols. An instrument for continuous monitoring of tidal volume (TVAM) has recently been incorporated into the dog inhalation exposure equipment. This instrument enables us to determine the mean tidal volume (TV), the mean respiration rate (RR), the minute volume (MV), and the total inhaled volume for each dog during exposure.

Complete respiration data during both training and exposure have been gathered for 98 dogs and compared in order to determine the reliability of the estimates of inhaled volume of dogs in those instances where the data were not obtained during aerosol exposure. The results showed that, despite the apparently significant differences between the means of the respiration data gathered during training and during inhalation exposure, the TV and MV data were both significantly correlated at the 99% level. This was not consistently true for the RR data. Therefore, equations were derived to enable TV and MV values to be estimated from the training data when necessary.

INTRODUCTION

It is necessary to know the volume of air inhaled by an animal during an aerosol exposure in order to accurately determine the quantity of material available for deposition in that animal. For rodent exposures, a mean minute volume (MV in ℓ/min) based on published data has usually been used, while for larger animals, values have usually been based upon measurements made prior to the exposures. These pre-exposure estimates may not be

accurate for the aerosol exposure due to changes in respiration induced by the aerosol.

In only a limited number of experiments have respiration parameters of experimental animals been measured during exposure (e.g., 1-4). Morken⁽¹⁾ used pressure capacitance transducers to detect pressure changes which occur in the breathing apparatus of beagle dogs, and Morrow⁽²⁾ reported results obtained using this system. Boecker⁽³⁾ described an

exposure apparatus for beagle dogs incorporating a whole-body plethysmograph. His paper also incorporates a summary of the various methods that had been tried elsewhere. Bair⁽⁴⁾ used a calibrated respiratory sensor for beagle dog inhalation exposures for a while, but this was discontinued due to recurring problems with the solenoid valve used to separate the inspired and the expired air. Design of the system⁽⁵⁾ used for the measurements to be reported in this paper started at that time. The flow-sensing unit is a venturi through which the dog breathes, measuring the volume of each breath and the total volume inhaled from the time measurement commenced.

Since all the dogs that are subjected to aerosol inhalation exposures have their respiration parameters measured daily as part of their pre-exposure training, it seemed appropriate to compare the values obtained during exposure with those obtained during training with a view to possible application in those instances where the data were not obtained during the aerosol exposure. To our knowledge, no previous comparisons of this kind have been reported in the literature. The data to be discussed below were gathered in two separate facilities using similar techniques but, for the most part, different pieces of equipment. For example, the design of the dog exposure box was unchanged, but the exposure box used in the second facility was not the same box that was used in the first facility.

METHODS

Training

The dogs scheduled for exposure are placed in metabolism cages 2 weeks prior to their exposure date. All the training conducted in the first facility and that for the first 36 dogs exposed in the second facility was carried out with the equipment shown in Figure 1. The dog inhaled air through a wet test meter (WTM) and a **three-way** respiratory valve. This directed the exhaled air past a thermister which operated a respiration register on each exhalation. The reading on each of these two instruments was obtained at the beginning and the end of the test period, yielding the total volume inhaled and the total number of respirations in a known time.

Training with the equipment described above for the inhalation exposure was as follows:

- Day 1 - 10 min familiarization in a test box which is part of the respiration monitoring equipment. No mask was placed on the dog.
- Days 2-5 - 10 min in box with mask on. The first 5 min are allowed for acclimatization, while the dog's respiration rate and inhaled volume are monitored over the second 5-min period.

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FIGURE 1. Wet Test Meter Respiration Monitoring Equipment

Days 6-10 - 10 min in box with mask and rubber insert in the mask the same as is used during the inhalation exposure, with the respiration rate and inhaled volume over 5 min being monitored each day.

Day 11 - Exposure of dog.
No training was conducted over the weekends.

The final 32 dogs exposed in the second facility were trained using

the tidal volume air monitor (TVAM) instead of the WTM to make the measurements. This necessitated use of the rubber insert from day 1 instead of day 6 of the training period. Mean training respiration rates (RR-TR), tidal volumes (TV-TR), and minute volumes (MV-TR) were calculated from the data gathered over the last 4 days of training.

The mask, which is molded from fiberglass, and the rubber latex insert are shown in Figure 2. This also shows the venturi tube, machined from Delrin, through which the dog

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FIGURE 2. Venturi Tube, Fiberglass Mask, and Rubber **Latex** Mask Insert

breathes. A dog in position for **inhalation** from the exposure chamber is shown in Figure 3.

Exposure

All the respiration data gathered during the inhalation exposure of 98

dogs in two facilities and the **train-**ing respiration data gathered on the last 32 dogs exposed in the second facility were obtained using the TVAM. The system consists of the dog inhalation mask and venturi air velocity meter (Figure 2), with associated transducers and electronic equipment.

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FIGURE 3. Beagle Dog with Mask on in Position to Inhale Aerosol from Exposure Chamber through Venturi Tube

The breath number, the serial time from the beginning of the exposure, and the volume (in ml) of each breath are automatically printed out at the end of each breath. The total volume of air inhaled up to the moment is printed out whenever a button is depressed.

The exposure respiration parameters calculated from the digital printer records are the mean respiration rates (RR-EX), the mean tidal volumes (TV-EX), and the minute volumes (MV-EX) for each dog. Changes in respiration rate and the distribution of tidal volumes with respect to volume, etc. are also available from the above records. The system is calibrated

with the aid of a Harvard pump before, and at intervals during, each series of exposures and measures tidal volumes in the range 40 to 400 ml with an error of less than 5%.

Analysis

The data gathered in the different facilities have been analyzed separately. Simple correlations between the various training and exposure parameters were computed. Analyses of variance on the different sets of data, such as between TV-TR and TV-EX for facility 1 or between TV-EX (facility 1) and TV-EX (facility 2), were also carried out. In order to be able

to use the training data in cases where respiration parameters were not measured during exposures, linear regression analyses were conducted on the data where appropriate. All the above analyses employed standard statistical techniques.

RESULTS

A typical data set giving the respiration parameters measured during training and during inhalation exposure is presented in Table 1. In Table 2, the means and standard

TABLE 1. Mean Respiration Parameters of Dogs Measured During Training and During Exposure

Dog Number	Training			Exposure			Dog Wt. Kg.	Expos. Time min
	TV ^(a) ml	RR ^(a) #/min	MV ^(a) l/min	TV ^(a) ml	RR ^(a) #/min	MV ^(a) l/min		
37	115	15.4	1.77	154	16.1	2.47	10.0	10.63
38	116	21.1	2.44	151	21.3	3.22	8.5	8.13
39	123	36.7	4.51	151	16.7	2.51	13.0	15.27
40	157	21.2	3.33	239	15.9	3.79	11.5	4.40
41	139	21.6	3.00	258	8.4	2.17	13.5	17.67
42	128	24.1	3.09	95.6	27.6	2.64	10.0	20.16
43	108	10.5	1.13	137	13.6	1.86	9.0	10.10
44	171	18.0	3.07	173	19.0	3.30	9.5	1.42
45	103	15.0	1.55	142	15.8	2.25	8.5	4.25
46	97.9	23.7	2.32	87.5	15.6	1.36	8.0	7.33
47	165	21.5	3.54	354	12.0	4.26	12.0	2.08
48	107	19.2	2.06	148	16.7	2.47	10.0	2.52
49	151	17.3	2.62	222	11.2	2.48	13.0	2.78
50	151	15.3	2.30	169	11.0	1.86	15.0	9.78
51	120	20.3	2.44	185	12.6	2.33	11.5	13.70
52	93.7	11.1	1.04	152	12.3	1.88	8.0	13.28
53	205	16.2	3.26	284	8.2	2.33	13.0	3.18
54	192	14.1	2.63	156	14.3	2.23	12.0	3.50
55	103	14.5	1.49	93.1	14.2	1.32	9.5	5.07
56	130	18.8	2.45	75.7	14.3	1.08	10.0	6.28
57	96.6	17.3	1.60	75.5	16.1	1.22	10.5	19.00
58	134	22.4	3.01	105	11.8	1.24	11.0	16.60
59	83.1	18.3	1.52	82.8	14.0	1.16	8.5	18.80
60	155	20.9	3.25	218	18.0	3.92	12.5	6.55
61	156	20.3	3.17	137	14.3	1.96	11.5	19.25
62	94.6	25.8	2.43	130	17.5	2.28	10.5	16.77
63	140	19.1	2.63	104	19.0	1.98	10.0	33.50
64	179	19.8	3.43	129	22.0	2.83	9.0	17.75
65	123	23.1	2.79	105	13.5	1.42	7.5	40.00
66	143	20.6	2.89	173	15.2	2.62	9.5	18.23
67	103	20.9	2.16	155	13.1	2.03	12.5	34.62
Para- Meter #	1	2	3	4	5	6	7	8

a. Abbreviations defined in text.

TABLE 2. Means and Standard Deviations of Respiration Parameters Measured during Training and during Exposure

Parameter #	Respiration parameter	First Facility		Second Facility	
		31 Dogs		67 Dogs	
		Mean $\bar{x}$	S.D. S_x	Mean $\bar{x}$	S.D. S_x
1	TV-TR (ml)	182.05	67.34	121.27	38.78
2	RR-TR (#/min)	18.24	3.37	21.85	5.72
3	MV-TR (l/min)	3.189	0.907	2.606	0.956
4	TV-EX (ml)	127.13	73.98	176.39	64.14
5	RR-EX (#/min)	20.52	8.18	15.49	4.46
6	MV-EX (l/min)	2.410	1.181	2.602	0.820
7	Body Weight (Ideal) (Kg)	11.02	1.50	11.04	1.86
8	Exposure Duration (min)	11.59	8.41	10.89	8.74

TR - Training
EX - Exposure

deviations of the various sets of observations are presented. This shows that there were some relatively large differences between the observations made under various conditions, both between facilities and between training and exposure. The mean body weights and exposure durations for the dogs exposed in the separate facilities showed no significant differences.

For each of the two facilities, simple linear correlations were determined between each of the eight parameters. The results for the pairings of concern to us here are summarized in Table 3. The results for

the first facility show that the data gathered with the TVAM during inhalation exposures were significantly correlated ($p < 0.01$) with that obtained on the WTM system during training for all three measured respiration parameters.

The correlation coefficients between exposure and training data for the second facility were not significant at the $p < 0.01$ level for either tidal volume or respiration rate. Since the equipment used to measure respiration parameters during training had been changed from the WTM to the TVAM system after 36 dogs had been exposed in the second facility,

TABLE 3. Correlation Coefficients (R) between Parameters of Interest

Facility	Parameters Being Compared		Number of Data Points N	Correlation Coefficient R	Significance Level	
	X	Y			p < 0.05	p < 0.01
First	TV-TR	TV-EX	31	0.776	Yes	Yes
	RR-TR	RR-EX	31	0.601	Yes	Yes
	MV-TR	MV-EX	31	0.649	Yes	Yes
	WT	TV-EX	31	0.534	Yes	Yes
	WT	RR-EX	36	-0.193	No	No
	WT	MV-EX	43	0.302	Yes	No
	Exp. Time	RR-EX	36	-0.277	No	No
Second	TV-TR	TV-EX	67	0.286	Yes	No
	RR-TR	RR-EX	67	0.275	Yes	No
	MV-TR	MV-EX	67	0.392	Yes	Yes
	WT	TV-EX	67	0.504	Yes	Yes
	WT	RR-EX	67	-0.168	No	No
	WT	MV-EX	67	0.344	Yes	Yes
	Exp. Time	RR-EX	67	-0.049	No	No

it was decided to analyze the data separately for each exposure group. The results are presented in Table 4, from which it is seen that the first group of 16 dogs yielded results that differed from those of the remaining groups. The means of the training data ($\bar{x}$ = 91.79 for tidal volume and $\bar{x}$ = 1.80 for minute volume) for this group are significantly lower than those for the other groups, something that is not evident for the means of the exposure data ($\bar{y}$ values). Results for the 31 dogs for which the same equipment (the TVAM) was used to

measure the respiration data during training as was used during exposures, and the results for 51 dogs left after excluding the first group of 16, are also given in Table 4. The training and exposure data are significantly correlated ($p < 0.01$) for both these groups. It is possible that leakage or gumming up of the WTM system was responsible for the lack of significant correlations for the first group of dogs. No significant correlations were observed for respiration rates at the 95% level for any of the groupings.

TABLE 4. Correlations between Training and Exposure Respiration Data When Grouped According to Exposure Dates

No. of Dogs	Dogs Included in Analysis	Respiration Parameter Involved	Trainin		Exposure		Cor. Coef. R	Significance Level	
			$\bar{X}$	S_x	$\bar{Y}$	S_y		$p < 0.05$	$p < 0.01$
16	1-16		91.79	46.69	183.38	59.28	0.021	No	No
20	17-36		128.67	32.03	200.69	59.94	0.505	Yes	No
16	37-52	Tidal	127.85	24.75	176.13	65.68	0.674	Yes	Yes
15	53-67	Volume	135.82	36.86	134.87	57.16	0.671	Yes	Yes
31	37-67	(ml)	131.71	30.93	156.17	64.19	0.565	Yes	Yes
51	17-67		130.52	31.08	173.63	65.72	0.494	Yes	Yes
16	1-16		1.80	0.78	2.83	0.88	0.344	No	No
20	17-36		3.34	0.79	2.93	0.60	0.517	Yes	No
16	37-52	Minute	2.51	0.91	2.55	0.76	0.544	Yes	No
15	53-67	Volume	2.58	0.64	1.97	0.78	0.627	Yes	No
31	37-67	(l/min)	2.55	0.78	2.27	0.81	0.513	Yes	Yes
51	17-67		2.86	0.87	2.53	0.80	0.598	Yes	Yes

None of the correlations between respiration rates during training and during exposure were significant at the 5% level.

The data presented in Table 3 show that there is a significant relationship between both tidal volume and minute volume and the body weight of the dogs but that this was not true for respiration rate. We were also not able to establish a significant relationship between the dogs' respiration rates and their exposure times--rather surprising, since it was expected that respiration rates would decrease with time as the dogs became used to the exposure conditions.

One-way analyses of variance were run between various sets of data to establish the significance level of the apparent differences between their means. The results are presented in Table 5. It is clear that many of the differences are real; for example, the mean exposure tidal volumes and respiration rates are significantly different ($p < 0.01$) in the two facilities, while the mean minute volumes are not. The same differences occur between the training and exposure data in the second facility. Analysis of the data

TABLE 5. Analysis of **Variance** to Determine Significance of Difference Between Means of **Respiration** Parameters Obtained Under Different Conditions

Primary Condition	Secondary Conditions being Compared		Respiration Parameter Involved	No. of Dogs in Group		F Statistic	Significance Level of F
	X	Y		n _x	n _y		
First Facility	Training	Exposure	TV	51	31	17.316	p < .01
	"	"	RR	51	31	0.910	N.S.
	"	"	MV	51	31	7.098	p < .01
Second Facility	Training	Exposure	TV	68	67	34.405	p < .01
	"	"	RR	68	67	46.386	p < .01
	"	"	MV	68	67	0.002	N.S.
Training "	Second Facility	First Facility	TV	68	51	50.300	p < .01
			RR	68	51	8.355	p < .01
			MV	68	51	21.133	p < .01
Exposure "	Second Facility	First Facility	TV	67	31	11.177	p < .01
			RR	67	31	15.337	p < .01
			MV	67	31	1.066	N.S.
Training-Second Facility	#s 1-36 WTM-Trained	#s 37-68 TVAM-Trained	TV	36	32	5.296	p < .05
			RR	36	32	12.456	p < .01
			MV	36	32	0.197	N.S.
Exposure-Second Facility	#s 1-36 WTM-Trained	#s 37-67 TVAM-Trained	TV	36	31	5.938	p < .05
			RR	36	31	0.228	N.S.
			MV	36	31	10.634	p < .01

WTM = Animals trained using wet test meter system.

TVAM = Animals trained using tidal volume air monitor.

from the first two and the last two groups of dogs exposed in the second facility yielded no informative pattern.

DISCUSSION

The mean values for the various parameters given in Table 2 may be compared with the following values obtained by Bair and Park⁽⁴⁾ for 12 dogs having a mean weight of 10.1 kg:

TV = 178.4 ml, RR = 24.1/min,
MV = 4.30 l/min

Pickrell, et al.⁽⁶⁾ reported mean values for 39 unanesthetized beagle dogs of mean weight 9.1 ± 1.3 kg as:

TV = 220 ± 42 ml, RR = 24 ± 4 /min

Product of these two figures gives an approximate minute volume of 5.28 l/min. The two groups of dogs that we studied had mean weights slightly

greater than the above (11.02 ± 1.50 kg for 31 dogs and 11.04 ± 1.86 kg for 67 dogs), yet their minute volumes were considerably lower. During training, the mean minute volume was 2.79 l/min, and only 13 of 98 dogs had minute volumes greater than 4 l/min. During exposure, comparable values were 2.54 l/min and 8 of 98 dogs.

Despite the apparently significant differences between the means of the respiration data gathered during training and during inhalation exposure, it is clear that tidal volumes and minute volumes are significantly correlated at the 99% level for both facilities, excluding data for the first group of dogs trained and exposed in the second facility. This means that not only is the training routine worthwhile, but the results obtained during training can be used to predict what the dog would have in-

haled during exposure. The WTM used during the training of the first group of dogs trained in the second facility had apparently become dirty. Subsequent checks revealed that it had been cleaned before the second group of dogs was trained in the second facility.

Linear regression equations both with and without an intercept were fitted to the different sets of training and exposure data. For the first facility, the equations that gave the best fit of the predicted to the observed points were as follows, where Y refers to the exposure value and X to the training value:

Tidal Volume: $Y = (0.711 \pm 0.045) X$

Respiration Rate: $Y = (10.96 \pm 4.82)$

$+ (0.494 \pm 0.247) X$

Minute Volume: $Y = (0.809 \pm 0.035) X$

Figure 4 presents graphically the

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FIGURE 4. Tidal Volume During Exposure Versus Tidal Volume During Training- 51 Dogs, Second Facility

data and fitted regression equation between the exposure and training tidal volumes for the last 51 dogs exposed in the second facility. The limits given are the 95% confidence intervals about the fitted line. For both tidal volumes and minute volumes, regression equations without an intercept were used because those with an intercept had a coefficient that was not significantly different from zero.

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AN INSTRUMENT FOR RAPID DETERMINATION OF CONCENTRATION
OF ALPHA-EMITTING AEROSOLS FOR USE IN ANIMAL INHALATION STUDIES

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Studies to determine the biological effects and metabolism of inhaled $^{239}\text{PuO}_2$ in beagle dogs require alveolar burdens over a 1500-fold range from 2 nCi to 3 μCi to be deposited in unanesthetized dogs by aerosol inhalation. Aerosols with concentrations ranging from 0.1 nCi/l to 5 $\mu\text{Ci}/\text{l}$ were generated by nebulizing water suspensions of $^{239}\text{PuO}_2$. To facilitate control of alveolar deposition, an aerosol concentration monitor which is capable of rapidly determining alpha radioactive aerosol concentrations immediately preceding and during dog exposures was designed and fabricated. Successive aerosol samples of 0.5 to 4.0 liters are drawn through an absolute filter by a remote controlled, automatically timed solenoid in a vacuum line. The filter remains in its original position while, immediately following sampling, alpha particles are counted by a system comprising a zinc sulphide scintillator and a photomultiplier. The detector is separated from the filter paper which is contained in a plastic filter holder by an air tight mylar cover. Several samples can be accumulated on a single filter paper. The monitor is capable of measuring the concentration within 2 min after initiation of sampling with an error not exceeding 20%.

Studies to determine the biological effects and metabolism of inhaled plutonium in beagle dogs require that predetermined amounts of $^{239}\text{PuO}_2$ and $^{238}\text{PuO}_2$ be deposited in the animals assigned to each dose-level group in the experiment. Alveolar deposition of these aerosols ranges from 2 nCi to 3 μCi per dog. In order to obtain these desired depositions, aerosols are generated by nebulizing water suspensions of $^{239}\text{PuO}_2$ particles to yield aerosol concentrations ranging from 0.1 nCi/l to 5 $\mu\text{Ci}/\text{l}$. To facilitate control and prediction of alveolar deposition in each dog, it is

necessary that the aerosol concentration during exposure be known. Due to the difficulty involved in generating aerosols of known concentrations at a constant rate, aerosol concentrations differing from that desired by an order of magnitude are not unusual. Thus, rather large errors could be expected if calibration of the aerosol generator some time previous to its use were relied upon for prediction of aerosol concentration during the exposure session. In the past, the actual mean aerosol concentrations only became available when the filter paper samples taken during

exposure of a dog were analyzed, usually days later. Consequently, an alpha aerosol monitor which can rapidly determine the aerosol concentration either immediately preceding or during exposure of the animal was designed and fabricated.

METHODS

The aerosol monitor, as depicted in the block diagram in Figure 1, consists basically of the following components: The detector is an RCA 6199, 1.5 in. diameter photomultiplier tube, the window of which has been sprayed with a thin layer of zinc sulfide scintillating material and covered with a 1 mg/cm^2 thick light tight cover of aluminized mylar. The filter holder (Figures 2 and 3) is a lucite cylinder

containing a 47-mm diameter Millipore type HA filter paper. The filter holder on the active side of the filter paper is covered only with a 1 mg/cm^2 thick layer of aluminized mylar to make it air tight but still allow passage of alpha particles for counting by the scintillator tube. The arrangement of the filter holder and the scintillator tube is shown in Figure 4 and is such that the filter paper is less than 1 centimeter from the scintillator tube face. Thus, in this arrangement, an alpha particle must traverse about 1 cm of air and two layers of 1 mg/cm^2 thick aluminized mylar before reaching the detector. The detection efficiency for ^{239}Pu is between 10 and 20%.

The photomultiplier tube is powered at 900 volts by a high voltage power

FIGURE 1. Block Diagram of the Alpha Aerosol Monitor

FIGURE 2. Cross Section Diagram of Filter Holder for Alpha Aerosol Monitor

supply.* The signal pulses are fed through a high voltage DC filter network to a linear amplifier.** To eliminate the low voltage noise pulses from the PM tube and thus reduce the background count, the amplifier signal is then fed into a discriminator.^t The resulting processed signal is counted by an electronic counter.^{t†}

* Model 403 M, John Fluke Mfg. Co. Inc., Seattle, WA.

** Model Z 101, Cosmic Radiation Labs, Inc., Patchogue, NY.

† Model DLA-501A, Cosmic Radiation Labs, Inc., Patchogue, NY.

†† Model 522B, Hewlett Packard, North Hollywood, CA.

A control circuit in conjunction with an electronic timer* controls both the counting time of the electronic counter and the sample volume which passes through the filter by means of a remote controlled solenoid valve in the vacuum line.

The aerosol monitor was designed to enable successive samples to be taken on the same filter paper. This eliminates the necessity of quickly changing filter papers between samples, a difficult procedure since this

* Model N803, Hammer Electronics Co., Inc., Princeton, NJ.

A.

B.

FIGURE 3. (A.) Photograph of Lucite Filter Holder with Mylar Cover Partially Cut Away Exposing Membrane Filter. (B.) Rear View of Filter Holder Showing Wire Mesh Filter Support.

FIGURE 4. Diagram Showing Relative Position of Filter Holder and Scintillator Detector Tube

must be done within the potentially radioactively contaminated animal exposure chamber. As each successive sample is taken, the previous count rate (background contamination) on the filter paper is known and subtracted to determine the Net Count Rate of the latest sample.

Due to statistical random variation in the disintegration rate of any radioactive source, it is necessary to count the sample for a finite length of time to assure with some confidence an accurate determination of sample

activity. For example, suppose we have a sample of activity λ . It is desired that this activity be determined with an error less than $\pm 10\%$ at a level of confidence of 95%. This sample must be counted for a length of time which will depend on the disintegrations per unit time that are detected (i.e., counts per unit time). If there is a background count, either from true background radiation or, as in this case, from a previous sample being deposited on the filter, then the counting time must be increased

to assure the same error in the determination of the net sample activity.

It was found necessary to construct a graph from which, if the approximate expected aerosol concentration and the background count rate on the filter before the sample is taken are known, one can easily determine the length of time that the sample must be counted in order to determine with a certain level of confidence its activity within a given error.

Development of the Error Equation

It must be assumed that the approximate concentration of the aerosol is known. This concentration will be represented by the unknown X . The background contamination (B) before the sample is taken can be measured. The efficiency (P) of the zinc sulfide detector is usually between 10 and 20% under the present geometry conditions.

$$\begin{aligned} S &= \text{standard deviation} \\ &= \sqrt{n} \\ &= \sqrt{(Y + B)t} \end{aligned}$$

where n = total number of counts

Y = sample count rate

B = background count rate

t = duration of count (min)

Error (E) at the 95% confidence level is then:

$$\begin{aligned} E &= 1.96 \sigma \\ &= 1.96 \sqrt{(Y + B)t} \end{aligned}$$

It is desired that the maximum error (E) be a certain percentage of the net count, Yt

$$\text{i.e., } E = QYt$$

where Q is the percentage error.

$$QYt = 1.96 \sqrt{(Y + B)t}$$

$$t = \frac{(1.96)^2 (Y + B)}{(QY)^2}$$

substituting $Y = 2.22 \times 10^6 \text{ XVP}$

we have:

$$t = \frac{(1.96)^2 (2.22 \times 10^6 \text{ XVP} + B)}{Q^2 (2.22 \times 10^6 \text{ XVP})^2}$$

where X = aerosol concentration ($\mu\text{Ci}/\ell$)

V = sample volume (R)

P = efficiency of detector

$2.22 \times 10^6 = \text{disintegrations/min}/\mu\text{Ci}$

A computer program was written to calculate the values of t for all combinations of the values of X , V , B , P , and Q for each of the three concentration levels listed in Table 1. These values were then plotted on graphs similar to Figure 5. Note that in Table 1 the value of percentage error (Q) increases as the concentration level category decreases. This is possible because of a smaller contribution to the total desired error of 20% by the dead air space in the lower level categories due to the larger sample volumes. It is advantageous to let Q be as large as is acceptable as this will allow shorter counting times (t).

The problem of determining aerosol concentrations for these low-level inhalation studies is further compounded by the fact that expected concentrations for animal inhalation studies vary over a wide range from 0.1 nCi/ℓ to 5 $\mu\text{Ci}/\ell$. Since this range of aerosol concentrations is so large, it was

TABLE 1. Values of X, B, V, P, and Q from Which the Sample Counting Time Graphs Were Generated (a)

	High Level	Medium Level	Low Level
X, in $\mu\text{Ci}/\ell$	0.5 1.0 2.0 3.0 5.0 10 20 30 50	0.01 0.02 0.03 0.05 0.1 0.2 0.3 0.5 1.0	0.0001 0.0002 0.0003 0.0005 0.001 0.002 0.003 0.005 0.01
B, in counts/min.	5×10^4 1.0×10^5 2.0×10^5 3.0×10^5 5.0×10^5 1.0×10^6 2.0×10^6 3.0×10^6 5.0×10^6 1.0×10^7 2.0×10^7 3.0×10^7 5.0×10^7	2.0×10^3 3.0×10^3 5.0×10^3 1.0×10^4 2.0×10^4 3.0×10^4 5.0×10^4 1.0×10^5 2.0×10^5 3.0×10^5 5.0×10^5 1.0×10^6 2.0×10^6	2.0×10^1 3.0×10^1 5.0×10^1 1.0×10^2 2.0×10^2 3.0×10^2 5.0×10^2 1.0×10^3 2.0×10^3 3.0×10^3 5.0×10^3 1.0×10^4 2.0×10^4
V, in liter	0.5	1.0	4.0
P eff. ratio	0.2	0.2	0.2
Q percent error	0.1	0.15	0.18

a. See text for definition of terms

advantageous to break it down into three concentration level categories as follows:

High range - 0.50 $\mu\text{Ci}/\ell$ to 5 $\mu\text{Ci}/\ell$

Medium range - 0.01 $\mu\text{Ci}/\ell$ to 0.50 $\mu\text{Ci}/\ell$

Low range - 0.0001 $\mu\text{Ci}/\ell$ to 0.01 $\mu\text{Ci}/\ell$

In order to reduce the range of total count rates for samples in the three ranges, larger samples are taken

for the low range than for the high range concentration. On the other hand, taking too large a sample would take longer than would be tolerated of a device designed to give a rapid determination of aerosol concentration. The following sample values were decided upon:

High range - 0.5 liter of aerosol

Medium range - 1.0 liter of aerosol

Low range - 4.0 liters of aerosol

FIGURE 5. Examples of Count Time Graph

It would be desirable to take samples of smaller volume for the high and medium ranges in order to increase the number of samples that can be taken on one filter paper before the counter becomes saturated. However, there is a dead air space in the sample tube and filter holder which amounts to nearly 30 ml. If it is assumed that between samples all residual particles in the dead air space settle out on the walls of the sample tube or filter holder, then 30 ml of diluting air containing little or no activity must be considered in the

sample volume. If a total error of less than 20% is desired, of which less than half will be due to the dead air space, then the total volume of aerosol sampled must be ten or more times the dead air space, i.e., 300 ml. A 500-ml minimum sample is well within the 10% error margin.

Use of the Instrument

The expected mean aerosol concentration should be known prior to taking the sample. This figure is arrived at

through previous measurement of aerosol generation rates (AGR) for the particular aerosol generator being used and also from a previously determined relationship between AGR and observed mean concentration of generated aerosols as determined by continuous sample taken on membrane filters during the entire animal exposure. The appropriate graph (one of three covering the three concentration ranges) used to determine the total counting time for various expected aerosol concentration and background count rates is chosen with knowledge of the expected concentration. The background count rate on the filter paper is measured. An aerosol sample of volume, equal to that used in determining values of the particular graph, is drawn through the filter and then counted for a time (T) equal to or greater than that derived from the graph. The concentration is then computed by the following equation:

$$x = \frac{(2.22 \times 10^6)(C-B \cdot T)(100)}{P \cdot V \cdot T}$$

where x = aerosol concentration
(in $\mu\text{Ci}/\ell$)

C = total counts

B = background contamination on filter
previous to sample (in counts/
min)

T = total counting time (in min)

P = percentage efficiency of detector

V = volume of aerosol sample (in
liters)

This computation is done by an on-line computer.

DISCUSSION

Several possible sources of error in measuring aerosol concentration in addition to the allowable $\pm 20\%$ must be considered. First, the maximum aerosol sample volume taken is 4 liters at a flow rate of 1.2 liters/min. Thus, the time over which this sample is taken is 200 seconds. This is essentially an instantaneous sample compared to the time a dog might be exposed to the aerosol, sometimes as long as 60 min. During the course of exposure, the aerosol concentration can be expected to change due to changes in generation rate; thus, the average aerosol concentration to which the dog is exposed may differ from the concentration measured before exposure begins. Samples can be taken and counted during long exposures to help correct for this drift in aerosol concentration, thus reducing, but not eliminating, the discrepancy. During the exposure of the dog, two continuous samples are drawn through membrane filters which are later counted on a separate counting system. This provides a value for the average concentration during the exposure. These values for a group of dog exposures as well as the instantaneous values taken by the alpha aerosol monitor before each exposure began are listed in Table 2. Note that the magnitude of the discrepancy between the two types of measurements can be as high as a factor of two. This is due to

TABLE 2. Comparison of Aerosol Concentrations as Determined by Alpha Monitor Preceding Animal Exposure (AM) and by Membrane Filter Sample Taken During Entire Animal Exposure and Counted Postexperiment (MF) (Values are arranged in increasing concentration.)

MF Concentrations nCi/ℓ	AM Concentrations nCi/ℓ	Ratio AM:MP
1.64	1.18	0.72
1.69	1.97	1.17
2.03	1.30	0.64
2.30	3.10	1.35
2.33	1.50	0.64
2.58	1.30	0.50
3.13	1.97	0.63
5.30	6.10	1.15
6.35	5.35	0.84
6.74	3.50	0.52
7.06	6.38	0.90
7.16	7.77	1.09
7.43	7.20	0.97
8.08	8.90	1.10
11.3	19.7	1.75
15.1	15.5	1.03
181	260	1.44
229	260	1.14
251	130	0.52
260	380	1.46
271	420	1.55
288	180	0.62
301	380	1.26
317	380	1.20
345	650	1.88
395	620	1.57
567	437	0.77
665	300	0.45
2120	1180	0.56
2260	1620	0.72
2430	1320	0.54
2570	2980	1.16
2590	1360	0.53
2690	3800	1.41
3760	6120	1.63
3860	6280	1.63

Mean ratio = 1.03 ± 0.42

the fact that the samples were taken by the two systems at different times, thus exemplifying the changes in aerosol concentration which can occur with time. Figure 6 is a log-log plot of the aerosol concentration as determined by the Alpha Aerosol Monitor versus the concentration determined from the continuous membrane filter samples. The equation of this line was calculated to be:

$$\begin{aligned} \text{Log}_{10} (\text{Alpha Aerosol Concentration}) \\ = (0.9961 \pm 0.014) \text{Log}_{10} \\ (\text{membrane filter concentration}) \end{aligned}$$

demonstrating the high correlation between the two methods of measurement.

A second source of error is due to the dead time of the photomultiplier tube, the efficiency of which was found to decrease for high count rates. A graph of efficiency versus count rate or total activity on filter paper is shown in Figure 7. Taking into account this dependence of detector efficiency on count rate is necessary to assure a total error of less than 20% when calculating aerosol concentration.

FIGURE 1. Block Diagram of Tidal Volume Air Monitor

proportional electrical signal which is then integrated with respect to time. Total volume information is stored in a separate integrator and is available for print out upon demand. Tidal volume, breath number, and serial time, measured from the beginning of exposure, are automatically printed on a permanent record tape in digital form at the end of each breath. An analog strip chart recording of air flow through the venturi is also provided, aiding in definition of respiration patterns.

TRANSDUCER

The choice of transducer to convert the air mass flow or volumetric flow into a suitable electrical signal was given careful consideration. It must satisfy several constraints consistent with the physiologic, technical, and operational requirements of the experiment.

1. Resistance to air flow through the transducer will give rise to abnormal, labored breathing by the test animal. This can cause artifacts in the radioactive aerosol deposition patterns thus affecting the final experimental result. The venturi tube offers minimal resistance to air flow while providing a sufficient pressure differential.
2. The transducer must not change the aerosol concentration or particle size distribution of the aerosol by causing particles to plate, impinge, or precipitate out of the air stream. This was a problem

with transducers of the blunt orifice, vane, or **pitot** tube type, while not particularly bad with the venturi tube. Any particulate matter or moisture which does **plate** or condense on the transducer parts must not change the calibration of the meter. Output fluctuations from the hot wire anemometer and thermistor type transducers resulted from this problem.

3. The transducer must be easily calibrated in the environment of a radiation contaminated exposure chamber.
4. The dead air space within the animal mask and the transducer will change the aerosol concentration; thus, it must be minimal. The venturi tube and dog mask now being used has a maximum dead air space of 25 ml. This value is about 15% of the average beagle dog's tidal volume and is taken into account in calculations.
5. The connection of the dog to the transducer assembly must be quick, simple, and leakproof since this procedure is performed in a potentially contaminated exposure chamber. The present mask system, as shown in Figures 2 and 3, fulfills these requirements.

The Herschel-type venturi tube was chosen as the least compromising transducer. The pressure differential, $P_2 - P_1 = \Delta P$ (see Figure 1) is detected by a differential transformer, bi-directional pressure transducer* and

* Model 270, Sanborn Division, Hewlett Packard, North Hollywood, CA 91604

FIGURE 2. Side View of Aerosol Chamber, Venturi Tube, and Dog Mask (Mask is clamped to tube with a plastic "U" shaped clamp.)

FIGURE 3. Venturi Tube, Mask, and Dog

associated carrier preamplifier. This transducer has a linear range of -400 to +400 mm H₂O and can accurately detect pressure changes of less than 0.1 mm H₂O.

The TVAM system was required to measure tidal volumes ranging from 30 to 300 ml with respiration rates ranging from 10 to 35 per min.

Using these ranges of tidal volume and respiration rate and assuming a normal respiration pattern, the limits of peak mass air flow ($\dot{Q}_{peak}$) during breathing were calculated to be:

$$\begin{aligned}\text{Minimum } \dot{Q}_{peak} &= 30 \text{ ml/sec} \\ &\approx 1.1 \times 10^{-3} \text{ ft}^3/\text{sec} \\ \text{Maximum } \dot{Q}_{peak} &= 900 \text{ ml/sec} \\ &\approx 32 \times 10^{-3} \text{ ft}^3/\text{sec}\end{aligned}$$

The differential pressure equation for the venturi tube being used was mathematically calculated to be:

$$\Delta P = 0.045 \dot{Q}^2 \quad (1)$$

where ΔP is expressed in mm of H₂O
and $\dot{Q}$ is expressed in $10^{-3} \text{ ft}^3/\text{sec}$

The equation was empirically checked and found to be:

$$\Delta P = 0.07 \dot{Q}^2 \quad (2)$$

The mathematical equation contained no allowance for the 36% permanent pressure loss which was included in the empirical equation. Using equation (2), the limits of the peak differential pressure (ΔP_{peak}) were calculated:

$$\begin{aligned}\text{Minimum } \Delta P_{peak} &\approx 0.08 \text{ mm of H}_2\text{O} \\ \text{Maximum } \Delta P_{peak} &\approx 49 \text{ mm of H}_2\text{O}\end{aligned}$$

The TVAM system was designed to measure pressure changes within these limits.

If it would have been possible to design a venturi which would yield symmetrical pressure readings of air flow in either direction, tidal volume could be derived by integrating both inhaled and exhaled air and dividing by two. However, restrictions of minimum dead air space precluded such design. Thus, it was necessary to differentiate between the inhale and exhale portions of the respiration cycle. This was accomplished by taking advantage of the small permanent pressure drop across the venturi constriction. A third pressure tap was placed at P_3 (see Figure 1), and the pressure differential $P_3 - P_2$ was monitored by a second differential pressure transducer.

The permanent pressure drop $P_3 - P_2$ is dependent on flow direction and thus was used to separate inhalation and exhalation. This separation is done during the electrical processing of the pressure data. Pressure differential $P_3 - P_2$ was also used as a breath recognition mechanism for breath counting.

PROCESSING AND INTEGRATION OF VOLUME FLOW SIGNAL ($P_2 - P_1$)

Processing of the differential pressure signal ($P_2 - P_1$) continues in the Volume Integration Network (see Figure 1). From equation 2, it is observed that $\dot{Q}$ is proportional to $\sqrt{P_2 - P_1}$. A multiplier/divider* is

* Model 1076, Hybrid Systems Corp., Burlington, Mass. 01803

employed as a square root module which performs the square root function on the signal $P_2 - P_1$ yielding $K \sqrt{P_2 - P_1}$. The signal then passes through a gating circuit. The gating signal is derived from the Breath Recognition Network described below and allows volume information to pass through the gating circuit during inhalation only.

The signal is further amplified, then fed into an analog strip chart recorder.* It is also fed into a commercial voltage to frequency converter.** The resulting output is a pulse train, the frequency of which is proportional to the input voltage. The pulses are counted, essentially a digital integration process, and stored by two counter registers. If the TVAM is properly calibrated, the number stored in these registers will be equal to the volume in ml of air which passed through the venturi tube during inhalation only (signals during exhalation are blocked by the gating circuit). The first register records tidal volume and consists of three decade counters. It is capable of recording volumes up to 999 ml. The number stored in this register is automatically printed on paper by a digital printer at the end of each breath and is then automatically reset to zero. The second register records total volume and consists of five decades with a capacity of recording volumes up to 99,999 ml. The stored number can be printed out at any time during the

course of the experiment by manually depressing a total volume print-out switch. The register is manually reset following each experiment.

PROCESSING OF THE BREATH RECOGNITION SIGNAL ($P_3 - P_2$)

Processing of the differential pressure signal ($P_3 - P_2$) continues in the Breath Recognition Network (see Figure 1). The signal from the carrier preamplifier is fed into a zero crossing detector comprised of an easily saturated operational amplifier followed by a Schmitt trigger circuit. The zero crossing midpoint can be set by means of a DC bias adjustment in the amplifier. By setting this midpoint slightly to the exhalation side of zero, errors due to "noise" in the system when the dog is not breathing can be eliminated. Output of the circuit is zero when $P_3 - P_2$ is positive (during exhalation) and is +15 volts when $P_3 - P_2$ is negative (during inhalation). This is the gating signal which, when supplied to the gating circuit, will allow volume information to pass during inhalation only.

The gating signal is further processed by the noise suppression monostable which can be driven at a maximum frequency of 5 Hz. Its function eliminates multiple triggering of the breath counter when noise occurs around the zero crossing point.

The output pulse of the noise suppression circuit, which occurs at the end of each inhalation, is fed to a monostable multivibrator and to a three decade breath counting register.

* Model 322, Sanborn Division, Hewlett Packard, North Hollywood, CA 91604

** Model 260, Fidar Corp., Mountain View, CA 94040

The digital-printer* receives its print command from the print command circuit. It is triggered by the trailing edge of the noise suppression monostable waveshape which insures that all data has been collected in the registers before the printer is energized. At this time the numbers stored in the breath counting register and the tidal volume register and the time measured from the beginning of the experiment, supplied by the digital clock,** are printed on paper by the digital printer.

The print command pulse is also supplied to the automatic reset circuit which resets the tidal volume register to zero following each

* *Model 562A, Hewlett Packard, North Hollywood, CA 91604*

** *Model 958-2 (S6404A), Honeywell, Test Instruments Division, San Diego, CA 92112*

breath. The automatic reset circuit contains a monostable multivibrator which provides a 640 millisecond delay to assure that all data are printed before the rest pulse is initiated.

PERFORMANCE

Calibration is accomplished by attaching an adjustable, calibrated pump to the venturi tube. Connection is completed through an absolute filter to assure no escape of radioactive aerosol into the room through the pump. The pump is set to three tidal volumes (50, 100, and 200 ml) and two respiration rates (20 and 30 resp./min) thus providing minute volumes ranging from one l/min to six l/min.

Figure 4 is a graph showing the tidal volume output of the TVAM with

FIGURE 4. Graph Showing Tidal Volume Output of TVAM with Respect to Known Tidal Volumes Pumped Through Venturi Tube at Various Respiration Rates (Limits of tidal volume and respiration rates for the beagle dog fall within the shaded area.)

respect to known tidal volumes pumped through the venturi tube at various respiration rates. From measurements taken on 68 trained dogs, the limits of respiration parameters were chosen as representative of the beagle dog. These limits, listed in Table 1, are represented on the graph of Figure 4 by the shaded area. The TVAM is capable of measuring tidal volumes with-

in the shaded area of the graph with an error between +5 and -0 percent.

Also included in Table 1 is the number of dogs within the group of 68 whose respiration parameters fell beyond the chosen limits as well as the actual maximum or minimum observed parameter values. It can be seen that the parameters of all but a few dogs fell within the specified measurement range of the TVAM.

TABLE 1. Respiration Parameter Limits Observed in 68 Dogs ("Chosen limits" were selected as representative of the beagle dog.)

	<u>Tidal Volume (ml)</u>	<u>Respiration Rate (Per Min)</u>	<u>Minute Volume l/m</u>
<u>Observed Mean Value</u>			
Maximum	363	34.3	4.53
Minimum	75.5	8.2	1.08
<u>Chosen Limit</u>			
Maximum	300	35	4.00
Minimum	30	10	1.00
<u>Number of Dogs Beyond Chosen Limit</u>			
>Maximum	3	0	3
<Minimum	0	5	0

INHALATION HAZARDS TO URANIUM MINERS - BIOLOGICAL STUDIES

This project will determine the biological effects of daily exposures to controlled levels of potentially carcinogenic uranium mine air contaminants in large and small experimental animals. The continuing experiments involve exposure of hamsters and dogs to radon daughters and uranium ore dust. A closely related project supported by the National Institute for Environmental Health Sciences, HEW, supplements these studies to include exposures to diesel exhaust fumes and cigarette smoke. The HEW and AEC projects were integrated into one experimental design at the request of the funding agencies, and the results of the combined projects are described in this report. A previously separate project "Uranium Miners Exfoliative Pathology," which compares lung washing samples from dogs exposed to laboratory-produced uranium mine atmospheres with similar samples from uranium miners, is now a part of this project.

BIOLOGICAL EFFECTS OF LIFE-SPAN INHALATION EXPOSURES
OF HAMSTERS TO RADON DAUGHTERS, URANIUM ORE DUST,
AND DIESEL EXHAUST FUMES

Investigators:

B. O. Stuart, R. F. Palmer, and R. H. Busch

Technical Assistance:

J. C. Gaven, E. J. Rossignol, and H. G. Steele

Six groups, each consisting of 102 male hamsters, began one year ago to receive daily 6-hr inhalation exposures to laboratory air (controls), 1200 WL radon daughters, 1200 WL radon daughters with uranium ore dust, uranium ore dust alone, diesel engine exhaust (50 ppm CO), or 1200 WL radon daughters with uranium ore dust plus diesel engine exhaust. Sacrificed hamsters have shown respiratory tract lesions covering a spectrum ranging from normal lungs in control hamsters to metaplasia of bronchiolar-alveolar epithelium (cuboidal metaplasia or bronchiolization) in hamsters in the experimental groups. Accumulation of particulate-laden macrophages about small bronchioles and vessels appeared within a few months in almost all of the hamsters exposed to diesel engine exhaust alone or with radon daughters and uranium ore dust; and, after more than 5 months of exposure, in about 50% of the hamsters exposed to uranium ore alone or with 1200 WL radon daughters. Where such particle deposits were heaviest, they were frequently accompanied by septal cell hyperplasia. Cases of metaplasia in groups receiving 1200 WL radon daughters with uranium ore dust, uranium ore dust alone, or 1200 WL radon daughters with uranium ore dust plus diesel exhaust, began to appear after 5 to 6 months of exposures, increasing sharply thereafter; adenomatous lesions began to appear after 6 months.

These experiments were designed to study the biological effects of inhaled potentially carcinogenic constituents of uranium mine atmospheres in response to national concern over the increased incidence of lung tumors among uranium miners of the Colorado plateau. Previous studies showed that hamsters receiving life-span daily exposures to 600 WL of radon daughters plus a carnotite ore dust (15 mg/m^3)

developed metaplasia and adenomatous lesions with anaplastic cells in the bronchiolar-alveolar epithelium (Annual Report, 1970). The current Phase II studies (Table 1) consist of daily exposures (6 hr/day) to 1200 WL radon daughters with and without concomitant exposures to uranium ore dust (carnotite) and diesel engine exhaust. These exposures are expected to cause minimum life-span shortening

TABLE 1. Experimental Design for Hamster Studies

<u>Group</u>	<u>Number of Animals</u>	<u>Phase I Exposures^(a)</u>	<u>Phase II Exposures^(b)</u>
1(c)	102	Controls (laboratory air)	Controls (laboratory air)
2(c)	102	30 WL radon daughters	1200 WL radon daughters
3(c)	102	600 WL radon daughters	1200 WL radon daughters with uranium ore dust (carnotite, 15 mg/m ³)
4(c)	102	600 WL radon daughters with uranium ore dust (carnotite, 15 mg/m ³)	Uranium ore dust (carnotite, 15 mg/m ³)
5(d)	102	-----	Diesel engine exhaust
6(d)	102	-----	Diesel engine exhaust plus 1200 WL radon daughters with uranium ore dust (carnotite, 15 mg/m ³)

a. Began October, 1968

b. Began November, 1970

c. Supported by AEC

d. Supported by NIEHS

while allowing maximum exposure time for development of pulmonary pathology.

PROGRESS TO DATE

In the preceding Phase I studies, life-span exposures to 600 WL radon daughters or 600 WL with 15 mg/m³ uranium ore dust produced vesicular emphysema, alveolar septal rupture, inflammatory cell exudation, fibrosis, hyalinization, occasional hyperplasia, and metaplasia of the bronchiolar epithelium. These changes were more frequent and severe in the 600 WL with uranium ore group, which also developed adenomatous lesions with scattered anaplastic cells associated with areas of advanced fibrosis. None of

the hamsters showed frank pulmonary neoplasia, but the lesions seemed to be progressing to that end. It was decided to raise the radon daughter exposure levels in the Phase II studies to 1200 WL to accelerate the development of pulmonary pathology. Group 4, receiving daily exposure to the carnotite uranium ore, was included to define the separate contribution of this mine inhalation hazard to lung pathology. The concentration of 15 mg/m³ of carnotite uranium ore in these studies is similar to levels of up to 20 mg/m³ found in typical small operating uranium mines on the Colorado plateau. In order to generate the desired aerosols for Group 5

and Group 6 exposures, a three-cylinder, diesel engine of the type that powers trackless vehicles in uranium mines was fitted with cycled load and throttle control systems; and the exhaust was automatically diluted and controlled at 50 ppm CO for daily exposure of hamsters with and without 1200 WL radon daughters plus ore dust.

Individual hamsters in each group are sacrificed when moribund. After 1 year of exposure, at least 50% of the hamsters are dead in each exposure group, including the controls. About half of the dead animals have been examined histopathologically. Control hamsters or hamsters exposed to 1200 WL of radon daughters alone almost invariably possess lungs that are essentially free from gross lesions. Hamsters that have inhaled uranium ore dust or uranium ore dust with 1200 WL radon daughters for several months generally show a diffuse yellow-tan discoloration. Hamsters in Groups 5 or 6, that inhale diesel exhaust, begin to show small focal points of black particulate accumulation within the first month of exposures. These areas have increased in number and at later periods (>6 months of exposure) have expanded throughout the lungs. Depending upon the exposure regimen, marked differences in the stage of development of respiratory tract lesions have been observed in animals sacrificed at times up to 10 months of daily exposures. These changes cover a spectrum ranging from normal lungs in control hamsters to moderate metaplasia of bronchial and bronchiolar epithelium and adenomatous hyper-

plasia of bronchiolar-alveolar epithelium (cuboidal metaplasia or bronchiolization) in hamsters in various exposure groups. The most frequent finding is that of particle deposits (soot and/or uranium ore) in focally accumulated macrophages nearly always adjacent to small vessels and bronchioles. Figure 1 shows the location of carbon particulates in the lysosomes of a macrophage in the lung of a hamster exposed to diesel exhaust. Where such deposits are heaviest, they may be accompanied by septal cell hyperplasia. This pattern of macrophage accumulation appeared within a few months of exposures and has become more pronounced in 95% of the hamsters exposed to diesel engine exhaust, alone or with radon daughters and uranium ore dust (Groups 5 and 6). A similar macrophage accumulation became evident in about 50% of the hamsters that inhaled uranium ore alone or combined with 1200 WL radon daughters sacrificed after more than 5 months of daily exposures (Groups 3 and 4). Cases of hyperplasia and metaplasia of the bronchiolar epithelium began to appear at 5 to 6 months of exposures, with a sharp increase thereafter. Adenomatous lesions have appeared at exposure periods greater than 6 months. The pathology appearing in individual groups is described below.

Control animals (Group 1) have seldom shown respiratory tract pathology; only three of 26 hamsters examined have shown lung lesions, consisting of two showing a very slight

FIGURE 1. Electron photomicrograph of a Macrophage in the Lungs of a Hamster Exposed to Diesel Engine Exhaust, Showing Carbon Particulates in the Lysosomes (10,400X)

degree of bronchiolar epithelium hyperplasia and one case of acute edema.

Hamsters that received 1200 WL of radon daughters on laboratory air aerosols (Group 2) have shown four cases of lung pathology among 22 examined (18%); of these, two animals showed some focal accumulation of macrophages, one had bronchiolar epithelial hyperplasia, and one showed adenomatous hyperplasia.

Group 3 hamsters received daily 6-hr inhalation exposures to 1200 WL of radon daughters deposited on sub-micronic aerosols of carnotite uranium ore (unattached fraction of radon daughters <1%). Thirteen of these (70% of those examined) have displayed one or more types of respiratory tract lesions. There were 11 cases of particle accumulation in

macrophages; three hamsters showed septal cell hyperplasia; four showed bronchiolar epithelial hyperplasia (three in animals that received >6 months of exposure); one case of bronchopneumonia appeared; one metastatic carcinoma, primary unknown, appeared; two hamsters showed bronchiolar epithelial metaplasia, both in animals sacrificed after 8 months of daily exposures. Figure 2 shows macrophage accumulating near bronchioles with hyperplasia and early squamous metaplasia of bronchiolar epithelium in hamsters from this group.

Group 4 hamsters are exposed to 15 mg/m³ of carnotite uranium ore alone. About 55% of the 22 animals examined showed significant lung lesions. All of those with lung pathology showed particles in focally accumulated macrophages; three had

septal cell hyperplasia; five showed bronchiolar epithelial hyperplasia; two had bronchiolar epithelial metaplasia; and one had a slight focal adenomatous proliferation. Figure 3

shows macrophage accumulation, fibrosis, and early metaplasia in the lung of a hamster from this group.

The hamsters in Group 6 received diesel exhaust fumes, 1200 WL radon

FIGURE 2. Lung Section from a Hamster Exposed Daily for 6 Months to 1200 WL Radon Daughters with 15 mg/m^3 Uranium Ore Dust, Showing Considerable Consolidation, Composed of Macrophages and Hyperplastic Septal Cells (The epithelium of the bronchiole shows hyperplasia and early metaplasia.) (H&E 96X)

FIGURE 3. Lung Section from a Hamster Exposed Daily for 6 Months to 15 mg/m^3 Uranium Ore (Carnotite) (Macrophages containing particulate material are accumulated adjacent to a bronchus which exhibits early metaplasia.) (H&E 96X)

daughters, and uranium ore dust. Thirty-six of the 37 animals examined in this group showed marked pulmonary pathology. All animals showed focal accumulations of macrophages containing particulate material; there was one case of interstitial pneumonia; two animals showed necrotizing **vasculitis** of pulmonary vessels (associated with the heaviest deposits of particulate material). After 5 to 6 months of exposure, eight hamsters showed septal cell hyperplasia (alveolar lining cells); ten showed bronchial and/or bronchiolar epithelial (basal cell) hyperplasia; seven had developed bronchial and/or bronchiolar metaplasia; and three hamsters showed adenomatous hyperplasia (focal adenomatous lesions in the bronchiolar alveolar epithelium). Figure 4 shows heavy focal **accumula-**

tions of carbon in macrophages with hyperplasia of septal cells and **hyperplastic** epithelium lining a large **bronchiole** in a Group 6 hamster.

Group 5 hamsters are receiving life-span daily exposures to diesel exhaust alone (50 ppm CO). All animals examined histopathologically have shown focal accumulations of macrophages containing particulates. Percentage incidences for the various lesions were similar to those for Group 6 hamsters; among 14 animals showing lung lesions, four had septal cell hyperplasia, three showed bronchiolar epithelial hyperplasia, two showed focal **adenomatous** hyperplasia, and one had a lobar pneumonia. Figure 5 is a section of lung from a Group 5 hamster showing heavy deposits of carbon in **macro-**phages distributed throughout alveolar

FIGURE 4. Lung Section from a Hamster Exposed Daily for 6 Months to 1200 WL Radon Daughters 15 mg/m³ Uranium Ore Dust and Diesel Engine Exhaust (50 ppm CO) (Carbon, presumably in macrophages, is focally accumulated; alveolar septal cells adjacent to these deposits appear **hyperplastic**. The lung epithelium of the bronchus is metaplastic.) (H&E 204X)

FIGURE 5. Lung Section from a Hamster Exposed to Diesel Exhaust Only (50 ppm CO) for 6 Months (Heavy deposits of carbon are present in the macrophages diffusely distributed throughout the alveolar septa and focally accumulated adjacent to respiratory bronchioles and small vessels.) (H&E 204X)

septa and adjacent to a respiratory bronchiole and a small vessel.

The daily exposures described in the Phase II studies will continue throughout the life-span of these animals. Fifty-four additional hamsters have been added to each group. Hamsters from each of these groups will be sacrificed periodically to determine the progression of lung and

systemic organ pathology without the complication of morbidity and to provide tissue samples for radon daughter and long-lived radionuclide analyses to determine absorbed dose patterns. They will also provide animals for periodic lung washing to correlate exfoliative cytology with the observed pathology.

DISPOSITION OF INHALED RADON DAUGHTERS IN HAMSTERS**Investigators:**

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Technical Assistance:

J. C. Gaven and H. G. Steele

A dynamic simulation of deposition and translocation of inhaled radon daughters was developed to determine the dose rate and accumulated radiological doses resulting from daily life-span exposures of hamsters to controlled levels of single and combined uranium mine air contaminants. The simulation is fitted to measured chamber concentrations of specific radon daughters and to direct measurements of these radionuclides in the tissues of sacrificed animals. Absorbed dose patterns thus obtained permit the extrapolation of dose-effect relationships obtained in experimental animal studies to man.

INTRODUCTION

Groups of 102 hamsters each have been receiving life-span inhalation exposures to 1200 WL radon daughters, with and without concomitant exposures to carnotite uranium ore dust and diesel engine exhaust, to identify the agent or combination of agents and their doses required to induce respiratory and systemic organ pathology, in order to clarify the problem of lung cancer in uranium miners. The animals are placed in 3 m³ chambers for daily 6-hr exposures to simulated uranium mine exposures, causing exponential build-up of radon daughters

in the respiratory tract and systemic organs at the beginning of each exposure period. A dynamic simulation using hybrid computer facilities was set up to study radiological dose patterns developing in various tissues with repeated daily exposures and to examine the build-up of long-lived radon daughter radionuclides. The preliminary simulation was designed using the recommendations of the Task Group on Lung Dynamics (Health Physics 12, 173-200, 1966) and the suggested ICRP values for specific biological half-lives (Health Physics 3, 1-380, 1960) and is being modified as data on specific tissue levels of radionuclides become available from periodically sacrificed animals.

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PROGRESS TO DATE

The dynamic simulation of the behavior of radon daughters was developed using a hybrid computer consisting of an EASE 2133 analog computer for simultaneous integration of differential equations and a DEC PDP-7 digital computer for computation and data storage. The simulation consists of three sequential components that describe the steady-state equilibrium levels of radon daughters in the exposure chambers; the build-up of the short-lived (<1/2 hr) radon daughters RaA (^{218}Po), RaB (^{214}Pb), RaC (^{214}Bi), and RaC¹ (^{214}Po) in the respiratory tract and systemic organs; and the long-term accumulation of the long-lived daughters RaD (^{210}Pb), RaE (^{210}Bi), RaF (^{210}Po), and RaG (stable lead) in these tissues. The results of radon samples taken hourly over each day's exposures (6 hr/day) and daily determinations of specific radon daughter concentrations are used to verify and fit the simulation of radon daughter concentrations and flow patterns through the exposure chamber and specifically at the point of inhalation by the experimental animal. Differential equations of the form

$$\dot{A}_{n,i} = f_{1,j,n} \cdot \lambda_{b,j,n} \cdot A_{n,j} + \lambda_n \cdot A_{n-1,i} - \lambda_{e,i,n} \cdot A_{n,i}$$

are used to describe the pulmonary deposition and translocation of a given radionuclide from one compartment (or organ) to another; $\dot{A}_{n,i}$ is the instantaneous level of radionuclide "n" in organ "i". The first term represents the accumulation in "i" from organ "j"; the second term is the accumulation by radioactive decay in organ "i" from the

parent radionuclide; and the last term is the loss of $A_{n,i}$ from organ "i" by the effective decay (radioactive plus biological). The equations for the three compartments of the respiratory tract (nasopharynx, tracheobronchial, and pulmonary as shown in Figure 1) have an additional term of the form $D_n \cdot \dot{V}_n$, where D_n is the deposition fraction and $\dot{V}_n$ is the hour-volume of respiration. In Figure 1 the values of D_3 , D_4 , and D_5 are the percentages of the total inhaled aerosol that are deposited in each region; biological half-lives for each compartment or tissue are shown. Figures 2 and 3 illustrate the behavior of these radionuclides in representative tissues; dynamic representations of radioactivity levels following repeated daily

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FIGURE 1. Block Diagram of the Biological Disposition of Inhaled Radon Daughters (D_3 , D_4 , and D_5 are the percentages of the inhaled radon daughters deposited in nasopharyngeal, tracheobronchial, and pulmonary regions of the lung.)

exposures of radon and its short-lived daughters in the pulmonary compartment of the respiratory tract and the liver are shown. This simulation is being fitted to experimentally determined levels of specific radon daughter radionuclides in tissues obtained immediately after sacrifice of hamsters exposed for varying periods of time. Preliminary results indicate that the respiratory tract deposition fraction may vary significantly between individual animals. The experi-

mentally verified model allows determination of absorbed dose to various lung regions and to systemic organs as a function of radon daughter levels and varying free ion fractions with exposures to radon daughters alone and in the presence of uranium ore dust and diesel engine exhaust; it also affords a means of extrapolating observed small and large experimental animal dose-effect relationships to man.

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FIGURE 2. Dynamic Simulation of the Radioactivity Levels of Radon (C_R) and Its Daughters (A_A , A_B , A_C , A_D) in the Pulmonary Lung During Repeated Daily Exposures to 1200 WL [C_R = inhaled concentration of radon ($\mu\text{Ci}/\text{l}$); A_A , A_B , A_C , A_D = deposited radioactivity level (μCi) of each radon daughter.]

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FIGURE 3. Dynamic Simulation of the Predicted Radioactivity Levels of Radon Daughters Translocated to One of the Systemic Organs, Including Build-up of Long-lived RaD (^{210}Pb) with Daily Exposures to 1200 WL (Ordinate scales of radioactivity are in μCi units.)

BIOLOGICAL EFFECTS OF LIFE-SPAN INHALATION
EXPOSURES OF BEAGLE DOGS TO RADON DAUGHTERS,
URANIUM ORE DUST, AND CIGARETTE SMOKE

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Sixty beagle dogs are continuing to receive daily inhalation exposures to 600 WL radon daughters with uranium ore dust and/or cigarette smoking (10 cigarettes/day, 7 days/week). After 2 years of exposure, respiration rates and volumes and body weights have shown no sustained differences between the exposure groups and controls. Total white blood cell counts and segmented neutrophil levels rose significantly during the last 6 months in the group receiving 600 WL radon daughters with ore plus cigarette smoking. Lung lavage samples from dogs in this group and in the group receiving 600 WL radon daughters with ore dust show large macrophages with increased numbers of neutrophils and occasional lymphocytes. The lungs of four dogs (one per group) sacrificed 6 months after beginning exposures showed no abnormalities in the control dog but perivascular and peribronchiolar accumulations of macrophages in the dog inhaling cigarette smoke. The dogs receiving 600 WL radon daughters with ore dust, with and without cigarette smoking, showed peribronchiolitis, perivascularitis, fibrosis, vesicular emphysema, and hyperplastic and metaplastic lesions of the bronchiolar epithelium.

The incidence of lung cancer among the uranium ore miners of the Colorado plateau is six times that observed in nonminers, and additional cases arise each year among these men. While the short-lived, alpha-emitting daughter products of radon are generally regarded as the primary carcinogenic agent, the miners are exposed to a variety of inhalation hazards including uranium ore dust and exhaust fumes from diesel engines. In addition,

nearly all of the miners who have developed lung cancer have had histories of cigarette smoking, and the possible synergistic or even primary action of cigarette smoking by uranium miners in the development of the observed lung cancers remains a controversial issue.

Experimental studies were undertaken 2 years ago using daily inhalation exposures of beagle dogs to known levels of mine air contaminants

separately and combined with daily inhalation of cigarette smoke to determine exposure-effects relationships.

PROGRESS TO DATE

Two groups of 20 dogs each are continuing to receive life-span, daily exposures for 4 hr daily to 600 WL of radon daughters with 15 mg/m³ of uranium ore dust with and without concomitant smoking of ten cigarettes over a 16-hr period. Twenty-nine dogs in the control group and the non-smoking radon exposure groups receive identical periods of sham smoking using unlighted cigarettes. After nearly 2 years of daily exposures, there have occurred no sustained differences in monthly mean body weights, minute volumes, tidal volumes, or

respiration rates between dogs in exposed and control groups.

Blood samples are taken quarterly from each dog. Erythrocyte and hemoglobin levels in all groups are not significantly different from controls. Total white blood cell counts and absolute segmented neutrophil levels were significantly higher after 18 and 22 months exposure in the group receiving radon daughters with ore plus cigarette smoke as shown in Figure 1. The changes apparent in the exfoliative cytology of these dogs (described elsewhere in this report) are such that the neutrophilia may be explained as a response to chronic irritation and inflammation in the lung. All other white blood cell levels, including lymphocytes,

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FIGURE 1. Mean Neutrophil Levels in Peripheral Blood, with 95% Confidence Limits as Indicated

eosinophils, monocytes, and banded neutrophils, remain not significantly different from controls.

Routine analyses of serum constituents obtained quarterly from each dog were performed to determine levels of urea nitrogen, creatinine, glucose, alkaline phosphatase, SGOT, and SGPT. No consistent differences have appeared between mean control and experimental values, other than occasional high SGPT values in individual animals in each exposure group. No significant differences from controls were observed in mean thyroxine levels, but values for individual dogs in both smoking groups were generally lower than for nonsmoking dogs.

Periodic physical examinations have shown no serious abnormalities in these dogs. Periodic radiographs of all dogs have shown only occasional transient diffuse densities in the lungs of individual animals in the experimental groups.

The previous report briefly described the pathological findings in

the lungs of four dogs (one from each group) sacrificed 6 months after beginning exposures. No abnormalities were found in the control dog. The dog receiving daily inhalation of cigarette smoke showed perivascular and peribronchiolar accumulations of macrophages and lymphocytes. The lungs of the dog receiving 600 WL radon daughters with ore dust showed hyperplastic and metaplastic changes in the bronchiolar epithelium, diffuse fibrosis, peribronchiolitis, and perivascularitis. The lesions found in the lungs of the dog that inhaled radon daughters with ore plus daily cigarette smoking were quite similar, including scattered and focal metaplastic lesions, vesicular emphysema, and focal areas of fibrosis. Histopathologic examination was conducted on tissues from a dog that died unexpectedly 21 months after beginning daily exposures to cigarette smoking and radon daughters with ore. Figures 2 and 3 show lung sections of

FIGURE 2. Lung Section from a Beagle Dog Exposed for 21 Months to 600 WL Radon Daughters with 15 mg/m³ Uranium Ore Dust and Cigarette Smoke (Macrophages containing particulate material have accumulated around small vessels.) H&E 320X

FIGURE 3. Lung Section from the Dog Described in Figure 2 (Particulate-containing macrophages have accumulated adjacent to small bronchioles.) H&E 320X

this dog containing macrophages laden with particulate material that has accumulated around small vessels (Figure 2) and adjacent to small bronchioles (Figure 3). Figure 4 shows a section from the same lung having macrophages containing particulate material, together with fibroblasts and a few small mononuclear cells adjacent to a small bronchiole.

In addition to these focal locations, macrophages were diffusely distributed throughout the lungs. The lung lesions present in this animal showed little evidence of advancement or increase in severity compared to its counterpart sacrificed after 6 months of exposure. There was no apparent reason for this acute death.

FIGURE 4. Lung Section from the Dog Described in Figure 2 Above (Macrophages laden with particulates, plus fibroblasts and a few small mononuclear cells are located adjacent to a small bronchiole.) H&E 96X

EXFOLIATIVE LUNG CYTOLOGY OF BEAGLE DOGS EXPOSED TO
RADON DAUGHTERS, URANIUM ORE DUST, AND CIGARETTE SMOKE

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Samples of deep lung cellular material were obtained by pulmonary lavage from beagle dogs exposed to radon daughters with U ore dust plus cigarette smoke, radon daughters with U ore dust, cigarette smoke only, and control dogs. The samples were obtained for the purposes of technique evaluation and cellular detail studies.

Comparisons of cell-counting techniques for determining absolute and differential counts were made. Influences on these counts due to method of preparation were studied.

Differential cell counts obtained by the methods regarded as most accurate indicated that dogs which were exposed to U ore dust had chronically inflamed lungs while those exposed to only cigarette smoke showed slight evidence of inflammation.

Samples of deep lung cellular material were obtained by pulmonary lavage from beagle dogs exposed to radon daughters with U ore dust plus cigarette smoke (two dogs), radon daughters with U ore dust (two dogs), cigarette smoke only (two dogs), and from two control dogs. Details of exposure including intervals, dose levels, have been previously described (Annual Report, 1970). The collections were made at 4-month intervals to develop studies of the morphologic characteristics of exfoliated pulmonary cells and to characterize changes in the morphology of these cells with continued chronic exposure to the aerosols.

Absolute cell counts, differential cell counts, and characterizations of cell morphology were determined from aliquots of each sample. Cell counts were obtained using the Coulter Counter, hemocytometer, and measured volumes of sample filtered onto the surface of millipore filters. The results obtained are summarized in Table 1. Counts obtained with the Coulter counter are higher than those obtained by other methods. These differences are related to the amounts of noncellular material present in the sample. Such material is more abundant in washings from dogs which received exposure to the aerosols, especially U ore dust, than in controls.

TABLE 1. Millions of Cells per ml of Lavage Fluid Recovered following Intratracheal Administration of 200 ml Saline

<u>Animal #</u>	<u>Treatment</u>	<u>Sample #</u>	<u>Coulter Counter</u>	<u>Hemocytometer</u>	<u>Millipore</u>	<u>Debris</u>
637	ROS	1	1.70	1.20	1.32	++++
		2	2.10	1.45	1.5	++++
629	ROS	1	1.70	0.90	1.02	++++
		2	1.50	1.10	1.30	++++
615	RO	1	2.50	1.50	1.40	++++
		2	4.00	3.20	3.00	++++
610	RO	1	4.00	1.50	1.56	++++
		2	2.60	1.05	0.98	++++
620	S	1	4.00	3.01	3.00	+++
		2	2.80	2.01	1.95	++
599	S	1	1.60	1.01	1.00	++
		2	2.80	2.20	2.08	++
628	C	1	1.20	1.90	1.00	+
		2	1.80	1.70	1.55	--
604	C	1	2.05	1.82	1.77	--
		2	1.00	1.10	1.05	--

ROS = radon daughters with U ore dust plus cigarette smoke

RO = radon daughters with U ore dust

S = cigarette smoke

C = controls

Smear preparations on glass slides and millipore preparations were used to determine differential cell counts. Smears on plain and frosted glass slides were prepared from pellets obtained by centrifuging measured volumes of lavage fluid. Millipore preparations were made from identical volumes of the same fluids. Glass slide preparations were stained by Papanicolaou's method (modified) and with new methylene blue, a stain directly applied and coverslipped without dipping or rinsing in volumes of solvents and dyes. Millipore preparations were stained with the modified Papanicolaou's method only. The results are summarized in Table 2.

The results suggest that cell loss during Papanicolaou's method of staining glass slide smears is selective. Macrophages and alveolar cells are retained better than small mononuclear cells, polymorphonuclear leucocytes, or other cell types including bronchial epithelial cells.

The contents of dye and solvent vats were centrifuged following staining of each type preparation and cell counts were determined by direct counting methods on resuspended pellets. The results are summarized in Table 3. These results show that most cells are lost during the first step consisting of immersion of the slides in water. The number of cells

TABLE 2. Cell Types by Percentage of Total Population in Lavage Fluid

Dog #	Treatment (a)	Macrophages Alveolar Cells					Small Mono- Nuclear Cells					Polymorphonuclear Leucocytes					Epithelial. and not Identifiable Cells				
		Method (b)					Method (b)					Method (b)					Method (b)				
		1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
637	ROS	65	72	43	40	45	34	26	50	51	53	1	2	6.4	8	1.5	0	0	0.6	1.0	0.5
629	ROS	75	60	37	35	40	23	35	48	52	50	1	5	6	5	3	1	0	9.0	8.0	7.0
615	RO	81	60	52	48	55	15	28	35	35	30	4	8	8	13	10	0	4	5	4	5
610	RO	50	41	27	29	31	48	56	62	60	60	2	2	8	10	8	0	1	3	1	1
620	S	86	80	75	71	78	14	13	15	20	11	0	1	4	5	3	0	6	6	4	8
599	S	89	71	70	66	69	7	20	18	16	20	4	7	11	16	9	0	2	1	2	2
628	C	75	75	70	70	72	25	24	29	30	27	0	0	0	0	0	0	1	1	0	1
604	C	80	80	70	70	68.5	20	17	21	25	21.5	0	3	5.3	3.5	6.0	0	0	4.7	1.5	4.0

- a. Method 1. Plain glass slide, Papanicolaou's stain
 Method 2. Frosted glass slide, Papanicolaou's stain
 Method 3. Plain glass slide new methylene blue
 Method 4. Frosted glass slide new methylene blue
 Method 5. Millipore filter, Papanicolaou's stain
 b. ROS = radon daughters with U ore dust plus cigarette
 smoke
 RO = radon daughters with U ore dust
 S = cigarette smoke
 C = controls

Table 3. Cell Content of Solvent and Dye Vats following Staining of Material on Glass Slides and Millipore Filters

<u>Step</u>	<u>Solvent or Dye</u>	<u>Cells per ml</u>		
		<u>plain glass</u>	<u>Frosted Glass</u>	<u>Millipore</u>
1	H ₂ O	30,000	15,000	110
2	Hematoxylin	1,100	0,350	0
3	H ₂ O	100	10	0

lost from millipore filters is relatively much smaller than from either type of glass slide. The frosted glass shows somewhat better retention than plain glass.

Data summarized in Table 1 indicate that cell counts of lavage fluid did not markedly vary with treatment group. The differential counts (Table 2) do seem to vary depending on treatment group with the greatest percentage of small mononuclear cells (lymphocytes and plasma cells) occurring in dogs receiving exposure to U ore dust (Figures 1, 2, and 3). The presence of these cell types indicates that the lungs of the animals receiving U ore dust exposure are chronically inflamed. Macrophage morphology also appears to change depending upon treatment. Macrophages from animals receiving U ore dust appear active as evidenced by large cell size, large indented nuclei, and/or multinucleation (Figure 1). Macrophages from animals receiving only cigarette smoke also show these characteristics but to lesser extent

(Figure 2). Macrophages from control animals retain their uniform shape and nuclear size (Figure 3).

Epithelial cells from dogs of all groups appear normal and represent only a very small fraction of the total cell population.

CONCLUSIONS

These studies indicate that the millipore technique is the best single method tested of preparing bronchial washing material for the purpose of determining numbers and types of cells present and providing material for morphologic study. The results of the studies on two washings from each of eight dogs suggest that the lungs of those which received U ore were chronically inflamed to some degree, while dogs which received exposure to cigarette smoke only showed slight evidence of such involvement. Epithelial cells appeared normal from all eight of the dogs examined.

FIGURE 1. Cells Filtered onto Millipore Filters from Pulmonary Lavage Fluid **Obtained** from Dog Exposed to Radon Daughters with U Ore Dust Plus Cigarette Smoke [A high proportion of small mononuclear cells (arrow A), reactive appearing **macrophages** (arrow B), and multinucleated cells (arrow C) characterize this cell population. 328X]

FIGURE 2. Cells Filtered onto Millipore Filter from Pulmonary Lavage Fluid Obtained from Dog Exposed to Cigarette Smoke [Small mononuclear cells make up a lesser proportion of the cell population than in Figure 1. Macrophages show some evidence of proliferative activity such as binucleation (arrow A) and size variation. 328X]

FIGURE 3. Cells Filtered onto Millipore Filter from Pulmonary Lavage Fluid from a Control Dog (Macrophages of fairly uniform size and shape are seen. Fewer small mononuclear cells are present than in Figures 1 and 2. 328X)

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SPACE NUCLEAR SYSTEM STUDIES

Space vehicles containing nuclear reactors or radionuclide power sources present special hazards in case of early abort or upon return from orbit. These problems are principally concerned with the biological effects of intensely radioactive small insoluble particles. We have studied and previously reported on the deposition and retention kinetics of inhaled $^{238}\text{PuO}_2$ particles and are retaining a number of dogs for observation of long term effects. The studies currently reported involved work with crushed $^{238}\text{PuO}_2$ microspheres such as have been employed in radionuclide power sources.

METABOLISM OF "SOLUBLE" ^{238}Pu FROM CRUSHED $^{238}\text{PuO}_2$
MICROSPHERES FOLLOWING INTRATRACHEAL OR INTRA-ABDOMINAL INJECTION

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Technical Assistance:

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"Soluble" ^{238}Pu , prepared by crushing $^{238}\text{PuO}_2$ microspheres, was administered to rats by intra-abdominal injection or intratracheal instillation. A large fraction of ^{238}Pu was excreted in the urine; urinary excretion of ^{238}Pu was markedly increased when DTPA was given with ^{238}Pu . Within hours, ^{238}Pu was translocated to the blood and concentrated in the skeleton. About 20% of intra-abdominally injected ^{238}Pu was initially deposited in the liver and mostly cleared during the first 40 days. Fecal excretion of ^{238}Pu was predominant following intra-abdominal injection whereas urinary excretion was greater following intratracheal instillation. About 70% of intratracheally instilled ^{238}Pu was cleared from the lung during the first day and an additional 20% during the next 40 days after instillation.

This report describes the metabolism of pulmonary or intraperitoneally deposited "soluble" ^{238}Pu obtained by crushing $^{238}\text{PuO}_2$ microspheres. The crushing procedure may be somewhat analogous to disintegration of $^{238}\text{PuO}_2$ as may occur with the stratospheric disintegration of missile-launched power sources.

OBSERVATIONS

Plutonium-238 microspheres were obtained from Mound Laboratory. Microspheres were 100-200 μm in diameter and had been prepared by the plasma torch process. Microspheres were thoroughly washed in water, lightly crushed with a mortar and pestle, and then washed ten times with 40 ml vol-

umes of sterile water with 1-min intervals between washings for settling out of particles. About 20 ml of upper supernatant were withdrawn following each washing. About 100 mCi ^{238}Pu , as lightly crushed, washed microspheres, were thoroughly crushed in 40 ml sterile water for 10 min with mortar and pestle. The microspheres were very fragile, easily disintegrating with little pressure on the pestle. The resulting suspension was allowed to settle for several hours. The upper 20 ml of this final suspension containing about 8 mCi ^{238}Pu were removed by syringe. This stock suspension of ^{238}Pu was diluted with saline for instillation or injection into animals.

The particle size of the ^{238}Pu was in the colloidal range, with a count median diameter of about 0.02 μm . Plutonium-238 for injection was examined on electron microscope grids. Distinct well defined particles were rare on the grids. Particles were difficult to size due to their lack of homogeneity in electron density and size and lack of distinct particle boundaries. Over 95% of the ^{238}Pu was eluted from 10 ml bed volumes of Sepharose 4B or 2B using 30 ml saline as elutant; 72% of ^{238}Pu in saline was dialyzed through Visking tubing with centrifugation of 700 rpm for 1 hr. Only 37% of ^{238}Pu sedimented in the bottom 3 ml (10 ml total volume) of saline following centrifugation at 3000 rpm for 3 hr. About 60% of ^{238}Pu in saline filtered through a GM-9 (Gelman) membrane filter.

Female, 80- to 100-day-old, Sprague-Dawley CD rats were used in this study. The experimental designs are shown in Table 1. Injections of 1.0 ml saline volume were given through the left mid-ventral lateral region of the abdomen in unanesthetized rats. Rats given intratracheal instillations of 0.3 ml saline volume were first anesthetized with halothane. External contamination of nose or mouth with ^{238}Pu was not found following intratracheal instillation.

Urine and feces were collected daily from rats placed individually in metabolism cages. All animals received food and water ad libitum. Serial sacrifices were performed on rats given intra-abdominal or intratracheal injections of ^{238}Pu . Tissues and organs were analyzed for plutonium contents. Selected tissues

TABLE 1. Experimental Designs for Study of ^{238}Pu Excretion and Tissue Distribution Following Various Routes of Administration

Route of Administration	^{238}Pu Administered (nanocuries)	Number of Animals	Time after Administration	Samples Taken
Intra-abdominal	1980 ^(a)	5	1-30 Days	Excreta + Tissues
Intra-abdominal	1980 + 0.5 ^(a,b) mmoles DTPA	5	1-30 Days	Excreta + Tissues
Intra-abdominal	1980 ^(a)	50	1 min - 35 Days	Tissues
Intratracheal	720 ^(c)	4	1-30 Days	Excreta + Tissues
Intratracheal	720 ^(c)	28	1 hour - 39 Days	Tissues

a. In 1 ml saline

b. Diethylenetriaminepentaacetic acid (DTPA) given with ^{238}Pu in same syringe

c. In 0.3 ml saline

were fixed in 10% NBF, embedded in paraffin, and autoradiograms performed on 6 μ m sections.

The amount and rate of ^{238}Pu excretion in urine and feces was dependent on the route of plutonium administration. The greatest amount of ^{238}Pu was excreted in the urine with 45% of intratracheally and 10% of intra-abdominally injected ^{238}Pu being excreted in urine the first day. By

the fifth day less than 0.1% of the initial dose was excreted daily in the urine of either group. Considerably more ^{238}Pu was excreted in the feces following intra-abdominal injection than following intratracheal injection, with 0.5-1% of the injected dose being excreted daily in feces during a 20-day period following intra-abdominally injected ^{238}Pu (Figures 1-2). About one-third of

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FIGURE 1. Excretion of ^{238}Pu following Intratracheal Instillation (Each point is a mean of daily plutonium excretion in urine or feces from four rats.)

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FIGURE 2. Excretion of ^{238}Pu following Intra-abdominal Injection of ^{238}Pu (Each point is a mean of daily plutonium excretion in urine or feces from five rats.)

the intra-abdominally injected ^{238}Pu was excreted in urine and feces during the first 30 days while more than 50% of intratracheally injected ^{238}Pu was excreted during this time (Table 2).

Intra-abdominally injected ^{238}Pu was rapidly cleared from the abdominal cavity into the blood starting at 1-2 minutes (0.1-0.2% of injected $^{238}\text{Pu}/\text{ml}$ blood) and increasing by 4-6 hours after injection to 1-2% of injected $^{238}\text{Pu}/\text{ml}$ blood. Activity in

the blood fell rapidly to less than 0.01%/ml blood by the tenth day. An estimated 25% of injected ^{238}Pu was present in the total blood volume at 4-6 hours after injection. Approximately 10% of administered ^{238}Pu was found in the liver and 6% in tissues of the gastrointestinal tract at 1-5 hours after injection. Plutonium-238 was rapidly lost from gastrointestinal tissues up to 2% of injected dose during the first day and then more slowly to 1% by 30 days. The

TABLE 2. Distribution of ^{238}Pu 30 Days after Administration to Rats (Values are percent of administered dose averaged for 4-5 rats.)

Organ or Tissue	Intra-abdominal (a)		Intratracheal (b)
	^{238}Pu	$^{238}\text{Pu} + \text{DTPA}$	^{238}Pu
Liver	5.4	0.24	1.7
Spleen	0.32	0.02	---
Gastrointestinal Tract	0.91	0.25	0.25
Kidney	0.21	0.02	0.12
Lung	0.07	0.01	14.0
Skeleton	40.7	4.8	19.1
Residual	12.9	1.5	5.3
Total Body Burden	60.4	6.8	40.4
Excreted in:			
Urine	11.6	79.8	48.8
Feces	24.2	9.1	3.9
Total Excreted	35.8	88.9	52.7
Amount Recovered in Excreta + Tissues	92.6	95.7	93.1

a. 1980 nCi ^{238}Pu initial deposition
b. 720 nCi ^{238}Pu initial deposition

liver burden was maintained at about 15% of injected ^{238}Pu from 1-9 days and then fell rapidly to 3-4% by 30-35 days after injection. Skeletal ^{238}Pu remained constant at 40% of injected dose from 1-35 days. The total body burden decreased by 10% the first day, by 20% the first 10 days, and by 40% the first 30 days

(Figure 3). Alpha tracks were individually spread throughout autoradiograms of cortical bone and concentrated on endosteal surfaces. They were slightly less uniform in liver, spleen, lung, and other tissues. There was little evidence of ^{238}Pu particles or aggregates as would have been indicated by stars on autoradiograms.

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FIGURE 3. Tissue Distribution of Intra-abdominally Injected ^{238}Pu

Intra-abdominal injection of ^{238}Pu with 0.5 mmoles DTPA greatly increased urinary excretion of ^{238}Pu , with about 75% of the injected dose being excreted during the first day.

Eighty-nine percent of the injected ^{238}Pu was excreted in urine (80%) and feces (9%) during the first 30 days after injection (Figure 4, Table 2). Only about 7% of initial

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FIGURE 4. Excretion of ^{238}Pu following Intra-abdominal Injection of ^{238}Pu and 0.5 mmoles DTPA (Each point is a mean of daily plutonium excretion in urine or feces from four rats.)

^{238}Pu remained in the rats at 30 days; 5% was present in the skeleton (Table 2).

Intratracheally instilled ^{238}Pu was cleared rapidly from the lung during the first day to about 30% of administered dose. Clearance continued more slowly during the next 38 days to about 10% of administered dose (Figure 5). Clearance of lung ^{238}Pu to blood was fastest during the first day with about 0.4-0.5% of instilled dose being found per ml

blood; this was reduced to about 0.02% at 20 days and to about 0.007% at 30-39 days. The ^{238}Pu content of liver remained at from 1.5-3.0% throughout the study period. About 5% of injected ^{238}Pu was found in tissues of the gastrointestinal tract at 1-5 hours, followed by a rapid loss of activity to about 0.2% at 30-39 days. Skeletal content of ^{238}Pu after the first few days amounted to about 20% of initially deposited ^{238}Pu . Total body ^{238}Pu decreased by

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FIGURE 5. Tissue Distribution of Intratracheally Instilled ^{238}Pu

about 10% during the first 6 hr, by 50% during the first day, and by about 65% at 30-39 days after instillation of ^{238}Pu (Figure 5).

CONCLUSIONS

Plutonium-238 was prepared for these studies by thoroughly crushing

$^{238}\text{PuO}_2$ particles in water, resulting in a ^{238}Pu fraction that behaved in rats like a "soluble" plutonium compound as well as exhibiting physical characteristics (filtration, dialysis, electron microscopic examination) indicative of ions or very small sized $^{238}\text{PuO}_2$ particles. Data on the metabolism and excretion of

this ^{238}Pu , indicating that the ^{238}Pu was largely in an effectively soluble form, were: (1) the rapid and significant excretion of ^{238}Pu in the first day's urine output; (2) the markedly increased urinary excretion of ^{238}Pu when given with DTPA; (3) the rapid translocation of intra-abdominally injected or intratracheally instilled ^{238}Pu from the site of initial deposition into the blood; (4) the concentration of ^{238}Pu in skeletal tissues and liver, irrespective of route of administration; (5) the significant fecal excretion of ^{238}Pu when given by intra-abdominal injection, presumably via bile, clearing a large portion of the liver ^{238}Pu

burden within 30 days; and (6) the diffuse distribution of single alpha tracks on autoradiograms of tissues.

The route of administration influenced the behavior of ^{238}Pu . Intra-abdominally injected ^{238}Pu was more heavily concentrated in skeleton and initially in blood and liver than was intratracheally instilled ^{238}Pu . More ^{238}Pu was excreted in the feces than in the urine following intra-abdominal administration whereas urinary excretion predominated following intratracheal administration. More ^{238}Pu was excreted within 30 days following intratracheal instillation than following intra-abdominally injected ^{238}Pu .

CARCINOGENICITY OF INHALED ^{238}Pu FROM CRUSHED MICROSPHERES

Investigator:

C. L. Sanders

Technical Assistance:

Thelma A. Jackson, Doris M. Meier, and G. J. Powers

An estimated 99% of inhaled "soluble" ^{238}Pu derived from crushed microspheres was cleared from the lungs of rats by 200-300 days after inhalation. About 50% of terminal ^{238}Pu was found in the skeleton. The highly toxic nature of ^{238}Pu was shown by increased mortality and tumor formation following initial lung depositions of 5, 18, or 207 nCi. To date 13-23% of animals exposed to ^{238}Pu and 1% of controls have developed nonmammary, mostly epithelial, tumors; 44% of nonmammary tumors were found in the lung following estimated cumulative radiation doses to the lung of 10 to 375 rads. Further interpretation awaits autopsy of remaining animals.

Little is known of the toxicity of inhaled ^{238}Pu . Plutonium-238 in a "soluble" form was prepared from crushed $^{238}\text{PuO}_2$ microspheres to simulate a form that might occur in fallout following stratospheric disintegration of missile-launched $^{238}\text{PuO}_2$ power sources. Rats exposed to this material by inhalation are still being observed, but preliminary results can be reported at this time.

OBSERVATIONS

Three groups of 34 female, Sprague-Dawley CD, 66- to 75-day-old rats were exposed to an aerosol of ^{238}Pu prepared by crushing $^{238}\text{PuO}_2$ microspheres in water and aerosolizing a small sized, "soluble" fraction. The procedures for the preparation and characterization of the Pu are described in the preceding report (this volume). Exposure times ranged from 10 to 30 min. Four rats from each group were autopsied at one day after exposure; the remaining 30 animals in each group were held for life-span observation. The mean amounts of ^{238}Pu measured in the lungs of the autopsied animals of each group at one day postexposure were 69.0 ± 43.6 , 5.9 ± 3.2 , and 1.6 ± 1.3 nanocuries ($\pm 95\%$ confidence intervals). The initial lung deposition of ^{238}Pu was estimated to be three times the amount present in the lung at 1 day following inhalation, based on intratracheal instillation studies with the same ^{238}Pu preparation, which demonstrated an approximate 67% loss of initially deposited ^{238}Pu from the lung during the first day after instillation (see preceding report). There were 108 un-

exposed control rats. All rats were given food and water ad libitum and housed in groups of 5-6 per cage. Animals were autopsied when dead or moribund. Erythrocyte and leucocyte counts were performed on cardiac blood obtained at autopsy from moribund animals. At autopsy, the total rat, except for the skull, was examined for tumor-like growths. Portions of all soft tissue organs, tumor-like growths, and other selected tissues were fixed in 10% neutral buffered formalin and paraffin sections prepared for microscopic examination following hematoxylin and eosin staining. The amount of ^{238}Pu present in tissues was determined by liquid scintillation counting. Autoradiograms were prepared from selected tissues.

Autoradiograms of lung sections at 1 day after inhalation of ^{238}Pu showed that the ^{238}Pu was mostly associated with the alveolar septae. Large plutonium aggregates, as evidenced by large alpha stars on autoradiograms, were rare. The alpha tracks were mostly randomly spread over the alveolar tissue at this time with few distinct stars comprised of more than four tracks. Occasional evidence of ^{238}Pu concentration of macrophages in bronchiolar lumens was also seen (Figure 1).

Based on studies of ^{238}Pu behavior following intratracheal instillation, from 60-70% of deposited ^{238}Pu is lost from the lungs within 24 hr after its deposition, and 90% by 40 days (Figure 2). Little of the injected ^{238}Pu was excreted in the feces, indicating that clearance from the respiratory tract via the ciliary escalator was not markedly involved.

FIGURE 1. Autoradiograms of Lung, One Day After Inhalation of ^{238}Pu (estimated initial ^{238}Pu deposition of 207 nCi) (Autoradiograms were exposed for three days. Upper: Alveolar region. Lower: Bronchiole containing ^{238}Pu laden macrophages. Both at 390 X magnification.)

Passage from the lung to the blood appeared to be the predominant process for removal of lung-deposited ^{238}Pu (see preceding report).

A small fraction of inhaled ^{238}Pu remained in the lungs at 300-500 days after exposure amounting to only 0.5-1.0% of the amount initially deposited. A trend towards loss of ^{238}Pu from the lungs at increasing time after exposure was apparent in each group with an estimated ^{238}Pu retention half-time in the lung of about 100-150 days from 300-500 days after exposure (Figure 2).

At 200-700 days after inhalation, about 1% or less of initially deposited ^{238}Pu was present in the lung, liver, or gastrointestinal tissues; and only 0.5% or less was found in spleen, kidney, or skin (Table 1). The high-

est amount of ^{238}Pu occurred in the skeleton, 38-67% of the terminal body burden or 8-18% of the initial body burden. Little ^{238}Pu appeared to have been excreted from the rats or translocated from the skeleton during the 200- to 700-day period. Approximately the same total body and skeletal plutonium burdens were found in autopsied rats during the first year of the study as during the second year of the study in each exposure group.

Hematology

Red blood cell counts in all groups inhaling ^{238}Pu were not significantly different from control counts. Both total leukocyte and lymphocyte counts were reduced with increasing deposition of ^{238}Pu . However, only in the

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FIGURE 2. Retention of Pulmonary Deposited ^{238}Pu in the Lungs of Rats [● Intratracheally instilled (720 μCi) ○ inhaled (5-207 nCi)]

TABLE 1. Distribution of Inhaled ^{238}Pu in Rats (Each value is a mean $\pm$ 95% confidence intervals of all autopsied animals at 200-700 days after inhalation.)

Tissue or Organ	Group I (207 nCi deposited)			Group II (18 nCi deposited)			Group III (5 nCi deposited)		
	nCi	% Terminal Body Burden	% Initial Body Burden	nCi	% Terminal Body Burden	% Initial Body Burden	nCi	% Terminal Body Burden	% Initial Body Burden
Lung	1.13 (11)(a) ± 0.65	3.47	0.55	0.15 (12)(a) ± 0.14	2.71	0.86	0.05 (14)(a) ± 0.04	4.56	1.01
Thoracic Contents	0.05 (13) ± 0.02	0.15	0.02	0.02 (14) ± 0.01	0.36	0.11	0.01 (14) ± 0.01	0.94	0.21
Liver	0.55 (17) ± 0.24	1.67	0.26	0.17 (18)	3.12	0.99	0.05 (19) ± 0.03	5.16	1.14
Gastrointestinal Tissues	0.16 (18) ± 0.05	0.49	0.08	0.10 (20) ± 0.04	1.78	0.56	0.07 (21) ± 0.04	6.60	1.46
Spleen	0.16 (18) ± 0.07	0.49	0.08	0.08 (20) ± 0.07	1.43	0.45	0.02 (19) ± 0.02	1.89	0.42
Kidney	0.11 (17) ± 0.06	0.33	0.05	0.05 (19) ± 0.04	0.89	0.28	0.03 (19) ± 0.02	2.83	0.63
Skin	0.59 (16) ± 0.54	1.80	0.29	0.10 (16) ± 0.05	1.78	0.56	0.03 (19) ± 0.01	2.83	0.63
Skeleton	22.20 (18) ± 4.40	67.91	10.72	3.23 (17) ± 0.82	57.64	18.26	0.41 (20) ± 0.15	38.24	8.45
Total in All Tissues and Organs	32.70 (18) ± 7.67	100	15.82	5.61 (20) ± 0.93	100	31.67	1.06 (21) ± 0.23	100	22.09

a. Number of animals sampled for each tissue or organ

TABLE 2. Hematologic Values for Cardiac Blood Obtained from Moribund Animals Following Inhalation of ^{238}Pu (Each value is a mean, $\pm$ 95% confidence intervals, of 8-10 samples from as many animals, obtained at 300-600 days after exposure.)

Group (a)	Red Blood Count ($10^6/\text{mm}^3$)	Leukocyte Counts ($10^3/\text{mm}^3$)	
		Total	Lymphocytes
Control	7.50 $\pm$ 0.61	7.74 $\pm$ 1.27	6.17 $\pm$ 1.25
4.8 nCi	7.85 $\pm$ 0.77	6.90 $\pm$ 1.55	5.12 $\pm$ 1.28
17.7 nCi	6.90 $\pm$ 1.01	6.39 $\pm$ 1.18	4.56 $\pm$ 1.00
207 nCi	7.56 $\pm$ 0.52	4.37 $\pm$ 1.74	3.02 $\pm$ 1.07

a. Estimated initial lung deposition of ^{238}Pu

highest exposure group (207 nCi) was the reduction significant (Table 2).

The median survival times of all groups exposed to ^{238}Pu were similar ranging from 550-600 days. The median survival time for unexposed control

animals was estimated at about 800 days (Figure 3).

The total incidence of tumors in all groups inhaling ^{238}Pu is presently about twice that observed in the control group (Table 3). However,

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FIGURE 3. Mortality of Rats Following Inhalation at ^{238}Pu (Animals were 66-75 days old when exposed to ^{238}Pu ; controls were 75 days old when placed on experiment. Actual age of animals is obtained by adding 66-75 days onto ages shown in figure. Animals are still alive in all groups.)

only about half of the control group has been autopsied while 75% of ^{238}Pu rats have been autopsied. Thus, the significance of much of this data awaits completion of the study. To date, there have been no large differences in the incidence of mammary fibroadenomas between controls and exposed animals; the incidence of mammary adenocarcinomas was greater in ^{238}Pu -exposed animals.

Other than mammary tumors, only one neoplasm has been seen in a control animal, a myeloid leukemia observed 700 days after being placed on experiment. The incidence of nonmammary

neoplasms in rats exposed to ^{238}Pu was considerably higher than in controls (Table 3), 23% following deposition of 207 or 18 nCi ^{238}Pu , 13% following deposition of 5 nCi. Of the 18 non-mammary tumors in all groups exposed to ^{238}Pu , 14 (78%) were of epithelial origin, originating in order of decreasing frequency in the lung (seven), uterus (two), and one each in the sebaceous gland, ovary, intestine, skin, and kidney. Of the non-epithelial neoplasms, one was a lymphosarcoma in the lung, one was a lymphosarcoma in the spleen, one was an adrenal medullary tumor, and one

was an osteogenic sarcoma of the lumbar vertebrae (Tables 3 and 4).

To date, nonmammary tumors have occurred from 345-699 days after exposure. The terminal ^{238}Pu body burdens in these tumor-bearing animals have ranged from 0.5 to 45 nCi.

Eleven of the nonmammary tumors were found in rats with body burdens of less than 8 nCi at death and seven in animals with terminal body burdens of 18-45 nCi. Of the eight lung tumors,

five were bronchiolar adenocarcinomas located as focal lesions in the lung periphery; one was a focal epidermoid carcinoma in the lung periphery; one was a squamous cell carcinoma growing in the lung and surrounding thoracic tissues with numerous secondary tumors in the pleural cavity, and one was a lymphosarcoma which had replaced about half the lung volume. In addition, the animal with osteogenic sarcoma exhibited numerous metastasis

TABLE 3. Summation of Tumors Found in Autopsied Rats Following Inhalation of ^{238}Pu (Each rat was assumed to have only one tumor of each type even though many animals had multiple tumors of mammary origin. Animals are still alive in all groups. The data are based on autopsies of 75% of all animals inhaling ^{238}Pu and 45% of control rats. The tumor incidences are based on all animals whether alive or dead.)

Tumor Location	Incidence of Tumors (%)			Unexposed Controls
	Group I (207 nCi deposited)	Group II (17.7 nCi deposited)	Group III (4.8 nCi deposited)	
Lung	10.0 (3) ^(a)	13.3 (4) ^(a)	3.3 (1) ^(a)	—
Sebaceous Gland	—	3.3 (1)	—	—
Uterus	3.3 (1)	—	3.3 (1)	—
Ovary	3.3 (1)	—	—	—
Intestine	—	3.3 (1)	—	—
Skeleton	3.3 (1)	—	—	—
Spleen	3.3 (1)	—	—	—
Skin	—	3.3 (1)	—	—
Adrenal	—	—	3.3 (1)	—
Kidney	—	—	3.3 (1)	—
Others	—	—	—	0.9 (1) ^(b)
Total: Non-mammary Tumors	23.2 (7)	23.2 (7)	13.2 (4)	0.9 (1)
Mammary Gland				
Adenocarcinoma	13.3 (4)	6.7 (2)	10.0 (3)	5.5 (6)
Fibroadenoma	20.0 (6)	33.3 (10)	40.0 (12)	22.2 (24)
Total	33.3 (10)	40.0 (12)	50.0 (15)	27.7 (30)
Total: All Tumors	56.5 (17)	63.2 (19)	63.2 (19)	28.6 (31)

a. Number of animals with tumor

b. Myeloid leukemia

TABLE 4. List of Nonmammary Tumors Found in Rats Following Inhalation of ^{238}Pu .

Tumor Type	Location	Time After Exposure (Days)	Terminal Body Burden (nanocuries)	Comments
Epidermoid Carcinoma	Uterus	345	1.1	—
Adenoma	Sebaceous Gland	347	3.5	—
Cystadenocarcinoma	Ovary	373	23.2	50 ml clear yellow fluid in abdomen; numerous secondary tumors on serosal surfaces.
Adenocarcinoma	Colon	385	3.7	Tumor occluded intestine; anal bleeding.
Osteogenic Sarcoma	Lumbar Vertebra	401	37.8	Hind legs paralyzed; Tumor metastasized to lung.
Bronchiolar Adenocarcinoma	Lung	429	1.1	Focal tumor in lung periphery.
Lymphosarcoma	Spleen	447	44.9	Splenomegaly; elevated blood lymphocyte count.
Carcinoma	Uterus	447	44.9	Same animal as above.
Bronchiolar Adenocarcinoma	Lung	520	7.2	Focal tumor in lung periphery.
Bronchiolar Adenocarcinoma	Lung	595	17.7	Focal tumor in lung periphery.
Squamous cell Carcinoma	Skin	599	4.5	Ulcerated area on dorsal part of body.
Epidermoid Carcinoma	Lung	601	5.8	Focal tumor in lung periphery.
Pheochromocytoma	Adrenal	653	1.2	—
Squamous cell Carcinoma	Lung	657	29.4	Two gram tumor in thorax; Infiltration in lung and around esophagus-trachea.
Bronchiolar Adenocarcinoma	Lung	688	3.5	Four focal areas of tumor in two lobes of lung.
Bronchiolar Adenocarcinoma	Lung	697	28.1	Focal tumor in lung periphery.
Transitional cell Carcinoma	Kidney	698	0.5	Markedly enlarged kidney with tumor arising from renal pelvis.
Lymphosarcoma	Lung	699	6.4	About half of lung volume is solid tumor mass.

in the lung. Photomicrographs of these tumors are shown in Figure 4. The cystadenocarcinoma in the ovary, lymphosarcoma in the spleen, and transitional cell carcinoma of the kidney were all large infiltrating tumors with secondary growths on the peritoneal serosa. Carcinomas were also seen in the colon, occluding its lumen, in the uterus, and in skin (Table 4).

Estimated mean radiation doses to lungs of rats at 500 days after inhaling ^{238}Pu were 10 rads, 340 rads, and 375 rads, following initial lung

depositions of 5, 18, and 207 nCi ^{238}Pu , respectively.

COMMENTS

Inhaled ^{238}Pu derived from crushed microspheres was not retained in the lung for a prolonged period of time; an estimated 90% of initially deposited ^{238}Pu was lost from the lung during the first 40 days after inhalation, and about 99% was lost by 200-300 days after inhalation. The retention half-time for ^{238}Pu in the

FIGURE 4. Pulmonary Tumors in the Lung Following Inhalation of ^{238}Pu

1.	Bronchiolar adenocarcinoma in lung 429 days postexposure.	167X
2.	Bronchiolar adenocarcinoma in lung 520 days postexposure.	399X
3.	Bronchiolar adenocarcinoma in lung 595 days postexposure.	399X
4.	Bronchiolar adenocarcinoma in lung 697 days postexposure.	399X
5.	Epidermoid carcinoma in lung 601 days postexposure.	167X
6.	Squamous cell carcinoma in lung 657 days postexposure.	167X
7.	Lymphosarcoma in lung 699 days postexposure.	399X
8.	Metastatic osteogenic sarcoma in lung 401 days postexposure.	167X

lung at 300-500 days after exposure was estimated at from 100-150 days. About 75% of initially deposited ^{238}Pu had been excreted during the first year after exposure. About half of the terminal ^{238}Pu in the body was found in the skeleton.

The highly toxic nature of inhaled ^{238}Pu was shown by the increased mortality and tumor incidences at initial

levels of deposition in the lung, ranging from 5-200 nanocuries. Of the nonmammary tumors, epithelial neoplasms predominated with the lung having the greatest number. Unexposed controls to date have not developed nonmammary epithelial tumors. Further interpretation of this study awaits autopsy of the remaining animals.

REMOVAL OF INTERNAL EMITTERS

This project is concerned with the development of methods which will hasten the removal, prevent the deposition, or otherwise decrease the damage potential from ingested or absorbed radionuclides. Associated with this effort is the development of information on the absorption and tissue distribution of radionuclides to help in understanding and predicting their behavior and to serve as a basis for recommendations for the treatment of exposed persons. Studies relating to the removal from the lung of inhaled radionuclides are covered in the Inhalation Studies project.

INTERACTION OF PLUTONIUM AND CERIUM DURING CHELATION THERAPY IN RATS

Investigator:

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Technical Assistance:

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In rats, during the early phases of deposition and decorporation by chelate therapy, an excess of stable cerium citrate had little effect on ^{238}Pu retention or removal by Zn-DTPA. On the other hand, an excess of ^{238}Pu relative to ^{144}Ce increased ^{144}Ce retention in the liver. Stable Ce administered as the DTPA chelate caused increased retention of ^{144}Ce in the soft tissues and was less effective in the removal of ^{238}Pu or ^{144}Ce than Zn-DTPA.

When rats were intravenously injected with a mixture of ^{238}Pu , ^{144}Ce - ^{144}Pr , and ^{65}Zn as the citrate complex (BNWL-1550, Part 1, p 97), the liver retention of Ce was elevated and the effect of DTPA (diethylenetriaminepentaacetic acid) on Ce removal was depressed over that usually seen in the absence of the other elements. Since the Ce was present in a lesser molar amount than the Pu, it seemed possible that a reversal of the relative amounts might affect Pu deposition and retention. It was also noted that ^{65}Zn was removed more effectively by Zn-DTPA than by Ca-DTPA. While the marked removal of ^{65}Zn by Zn-DTPA is a complex process, at least part of the effect is due to metal exchange. To test for the possibility of a common ion effect involving the nonphysiological element Ce and Ce-DTPA, groups of eight to ten rats were treated as shown in Table 1.

OBSERVATIONS

Techniques used were essentially the same as in the previous report (loc. cit.). All radioactive metals were administered intravenously as pH 4.6 citrates. Masses involved were low enough that nonradioactive Ce (^{140}Ce) and DTPA, when used, were present in molar excess. Results are shown in Table 1.

When used as a diluent, Ce appeared to increase Pu retention by the spleen and slightly decrease retention in the skeleton. When given as the DTPA chelate, Ce was not exchanged as rapidly for Pu as was Zn; and in the presence of excess Ce, removal of Pu by Zn-DTPA was slightly interfered with, all of which is in accord with the in vitro stability of the complexes. The large amount of ^{144}Ce retained in the liver when injected with Pu, at a molar ratio of 3000/1(Pu/Ce), is similar to that observed previously; however,

TABLE 1. Percent of Administered Radionuclides Retained by Rats 7 Days after Various Treatments

Intravenous Insult	Intraperitoneal ^(a) Treatment	Nuclide Measured	Percent of Administered Radionuclide($\bar{x} \pm s$)			
			Liver	Kidneys	Spleen	Skeleton ^(c)
1.69 $\mu\text{Ci } ^{238}\text{Pu}$	p.s. at 1 hr	^{238}Pu	16 $\pm$ 1.2	1.1 $\pm$ 0.09	0.46 $\pm$ 0.04	69 $\pm$ 3.0
1.69 $\mu\text{Ci } ^{238}\text{Pu}$	Zn-DTPA at 1 hr	^{238}Pu	1.4 $\pm$ 0.19	0.29 $\pm$ 0.032	0.20 $\pm$ 0.039	20 $\pm$ 2.4
1.69 $\mu\text{Ci } ^{238}\text{Pu}$	Ce-DTPA at 1 hr	^{238}Pu	9.7 $\pm$ 0.55	0.54 $\pm$ 0.023	0.87 $\pm$ 0.096	28 $\pm$ 2.1
1.69 $\mu\text{Ci } ^{238}\text{Pu}$	^{140}Ce citrate at 1/2 hr + Zn-DTPA at 1 hr	^{238}Pu	2.4 $\pm$ 0.16	0.28 $\pm$ 0.022	0.19 $\pm$ 0.033	24 $\pm$ 1.8
1.76 $\mu\text{Ci } ^{238}\text{Pu}$ + ^{140}Ce citrate ^(b)	p.s. at 1 hr	^{238}Pu	14.5 $\pm$ 1.0	1.6 $\pm$ 0.15	1.2 $\pm$ 0.15	55 $\pm$ 4.8
1.76 $\mu\text{Ci } ^{238}\text{Pu}$ + ^{140}Ce citrate ^(b)	Zn-DTPA at 1 hr	^{238}Pu	2.9 $\pm$ 0.31	0.40 $\pm$ 0.042	0.43 $\pm$ 0.075	27 $\pm$ 2.1
49.8 $\mu\text{Ci } ^{144}\text{Ce}$	p.s. at 1 hr	^{144}Ce	16 $\pm$ 1.1	2.2 $\pm$ 0.12	0.12 $\pm$ 0.0073	17 $\pm$ 1.3
49.8 $\mu\text{Ci } ^{144}\text{Ce}$	Zn-DTPA at 1 hr	^{144}Ce	6.4 $\pm$ 0.75	1.4 $\pm$ 0.095	0.086 $\pm$ 0.0076	11 $\pm$ 0.11
49.8 $\mu\text{Ci } ^{144}\text{Ce}$	Ce-DTPA at 1 hr	^{144}Ce	26 $\pm$ 1.1	2.8 $\pm$ 0.25	0.52 $\pm$ 0.060	13 $\pm$ 0.81
1.51 $\mu\text{Ci } ^{238}\text{Pu}$ + 52.0 $\mu\text{Ci } ^{144}\text{Ce}$	p.s. at 1 hr	^{238}Pu	8.8 $\pm$ 0.90	1.1 $\pm$ 0.12	0.32 $\pm$ 0.044	79 $\pm$ 4.2
		^{144}Ce	41 $\pm$ 1.6	2.7 $\pm$ 0.25	0.22 $\pm$ 0.034	18 $\pm$ 1.2
1.51 $\mu\text{Ci } ^{238}\text{Pu}$ + 52.0 $\mu\text{Ci } ^{144}\text{Ce}$	Zn-DTPA at 1 hr	^{238}Pu	1.0 $\pm$ 0.17	0.17 $\pm$ 0.016	0.069 $\pm$ 0.0076	26 $\pm$ 2.7
		^{144}Ce	2.8 $\pm$ 0.20	1.3 $\pm$ 0.05	0.12 $\pm$ 0.015	7.8 $\pm$ 0.28

a. Treatments:

p.s. = 1 cc physiological saline

Zn-DTPA = 1 mmol/kg, Zn salt of diethylenetriaminepentaacetic acid, pH 6.8

Ce-DTPA = 1 mmol/kg, Ce salt of diethylenetriaminepentaacetic acid, pH 7.1

Ce citrate = 5.0 $\mu\text{mol/kg}$, pH 7.0 (^{140}Ce is the stable form)

b. ^{140}Ce citrate = 1.6 μmoles , pH 4.6

c. Derived by using a femur factor of 22

no effect on removal effectiveness by Zn-DTPA or on skeletal retention was noted. The low liver retention of Pu in this case was not noted previously but is what is usually seen with an unusually high skeletal retention.

The common ion effect was detrimental in the removal of Ce and instead of operating in an exchange manner, as in the case of Zn, behaved as one would expect of a mass action effect increasing ^{144}Ce retention in the soft tissues. On the other hand, Pu exchanges reasonably well with

chelated Ce. Evidently Ce and Pu interfere somewhat with one another; but, in general, it is a chemical competition with the Pu-IV ion generally more effectively complexing with body ligands and DTPA. The high rate of exchange of chelated Zn with endogenous ^{65}Zn probably reflects the strength of the $\text{Zn}_2\text{-DTPA}$ chelate and physiological ligands and rapid exchange of the metal between metabolic pools; whereas, the behavior of ^{144}Ce and its low rate of exchange with chelated Ce appears to be more of an exchange between DTPA and ligands of

equal strength. This ignores the possibility that the Ce may not be in the intercellular fluid space and unavailable to the Ce-DTPA; but, since Zn-DTPA is able to remove Ce more effectively than Ce-DTPA, then contact is likely; and a purely chemical effect appears the more likely explanation.

CONCLUSIONS

The interaction between Pu and Ce

has little utility in preventing Pu deposition; and, at the mass levels studied, the deposition influences appear to be mediated by chemical means, e.g., mass action and ionic charge, rather than physiological effects. The common ion effect, so successful in preventing deposition of ^{65}Zn by administering Zn-DTPA, was not found for the nonphysiological nuclide ^{144}Ce and Ce-DTPA.

IN VITRO BINDING OF ^{239}Pu BY CaNa_3DTPA

Investigators:

J. E. Ballou and W. G. Morrow

DTPA enhanced the ultrafilterability of ^{239}Pu in blood serum and in a simulated lung fluid. The DTPA effect decreased rapidly after ^{239}Pu addition demonstrating the importance of prompt chelate therapy.

The effectiveness of DTPA (diethylenetriaminepentaacetic acid) in Pu removal is enhanced by prompt administration after Pu incorporation. Since the targets of early chelation therapy may include Pu in the lung or in blood, the availability of Pu for chelation was investigated at several time intervals after $^{239}\text{Pu}(\text{NO}_3)_4$ was added to a simulated lung fluid, to blood serum, or to blood plasma.

OBSERVATIONS

$^{239}\text{Pu}(\text{NO}_3)_4$ (5 μCi in 1 ml of 2 N HNO_3) was added to 100 ml of the solutions listed in Table 1; and, at timed intervals thereafter, duplicate 7 ml aliquots were measured into Visking bags for ultrafiltration. DTPA (25 mg) was added to one aliquot while the other served as the nontreated control. The ultrafiltrates were collected by centrifugation and analyzed

TABLE 1. Composition of Solutions Employed in ^{239}Pu Ultrafilterability Study

Gamble's Solution pH 7.9

<u>Salt</u>	<u>mg/l</u>
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$	0.212
NaCl	6.171
KCl	0.311
Na_2HPO_4	0.148
Na_2SO_4	0.079
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	0.255
$\text{NaC}_2\text{H}_3\text{O}_2 \cdot 3\text{H}_2\text{O}$	1.065
NaHCO_3	2.571

Gamble's Solution plus protein, pH 7.6

0.4% bovine serum albumin added to Gamble's solution

Human Plasma pH 6.8

Plasma separated by centrifugation from whole blood containing 67.5 ml ACD solution B in 450 ml of blood

Dog Serum pH 6.8

Serum separated by centrifugation from 500 ml of whole blood

for ^{239}Pu content. The ability to form the Pu-DTPA complex, as indicated by the percent ultrafilterability, should be related to the degree of hydrolysis of Pu or its strength of binding with constituents such as protein in the suspending solutions.

The results in Figure 1 show the time course of Pu availability for chelation in human plasma, dog serum,

and Gamble's solution, a buffered solution which stimulates the lung fluid environment. Plutonium-239 was consistently more ultrafilterable in human plasma than in the other media; however, DTPA had essentially no effect on this ultrafilterability. Since the plasma contained approximately 0.25% citrate as an anticoagulant, it is apparent that from 70 to 75% of the available ^{239}Pu was stabilized as the filterable citrate complex and only a small additional fraction could be complexed by DTPA. In dog serum, which contained no anticoagulant, ^{239}Pu was about 60% available for chelation after 5 min and progressively less chelatable up to 2 hr after ^{239}Pu addition. The decrease in ultrafilterability apparently reflects the hydrolysis of Pu and its chelate-resistant binding to serum proteins. It is interesting that roughly similar rates were observed with Gamble's solution whether or not protein was included. This suggests that Pu hydrolysis may be the primary action which interferes with the formation of the Pu-DTPA chelate. The greater DTPA effect in dog serum suggests that different mechanisms or states of Pu "inactivation" are involved in serum than in Gamble's solution.

CONCLUSIONS

These in vitro studies demonstrated a decrease in the availability of ^{239}Pu for chelation by DTPA in dog blood serum at a rate of 0.45%/min between 5 and 120 min after Pu addition.

FIGURE 1. Time Course of Plutonium Availability for Chelation in Human Plasma, Dog Serum, and Gamble's Solution

In a simulated lung fluid only one-third as much of the ^{239}Pu was available for chelation after 5 min, but the subsequent rate of decreased availability was approximately the same as for serum during the first hour after Pu addition. While the in

vivo situation is undoubtedly more complex, involving transport from the fluids as well as reaction with them, these results give some quantitative explanation for the importance of prompt chelation therapy.

THE BIOLOGICAL DISPOSITION OF $\text{Es}(\text{NO}_3)_3$ IN RATS AFTER INTRAVENOUS,
INTRAMUSCULAR, SUBCUTANEOUS, AND TRANSCUTANEOUS ADMINISTRATION

Investigator:

V. H. Smith

Technical Assistance:

Juanita S. Barnett and K. E. McDonald

Regardless of the injection route, $\text{Es}(\text{NO}_3)_3$ was retained preponderantly in the skeleton up to 24 days after intravenous (i.v.), intramuscular (i.m.), or subcutaneous (s.c.) administration. The liver burden decreased from 26% of the initial Es administered i.v. at 4 hours to 14% at 1 day and <2% at 24 days. The skeletal content increased to about 70% on day 7 and decreased to about 56% by day 24. The liver and skeleton retention at 24 days from the s.c. and i.m. injections was slightly less than from the i.v. route. Injection site retention was greater for the s.c. route, 16%, than for the i.m. route, 8%. Skin absorption was very high, about 4% of the available Es ($2.5 \mu\text{Ci}/\text{cm}^2$ pH 2 nitrate solution) being absorbed in 30 minutes.

The potential for accidental exposure to Es exists during production of ^{252}Cf and as it is studied in various laboratories. To be able to recommend treatment for an accidental exposure, preparatory studies of the biological disposition were undertaken to act as background information in interpreting treatment results and hazards. It was decided to use $\text{Es}(\text{NO}_3)_3$ in these studies because this is a likely to-be-encountered form and to compare with Pu and other actinides frequently encountered as nitrates. Einsteinium can be expected to behave more like a heavy lanthanide than Pu since it has only the +3 oxidation state in contrast to several oxidation states for the lighter actinides, and shielding by the 5f electrons in Es is more com-

parable to the type of shielding from 4f electrons encountered in the lanthanide elements.

OBSERVATIONS

$^{253}\text{EsCl}_3$, obtained from Oak Ridge, was converted to $\text{Es}(\text{NO}_3)_3$ by repeated evaporation with HNO_3 . The resulting material was kept in 6M HNO_3 and portions converted to a pH 2.0 solution for use as required. Using Visking membranes the $\text{Es}(\text{NO}_3)_3$ was shown to be at least 90% ultrafilterable. Groups of eight female, Hill-Top Wistar rats, 275-300 grams were injected intravenously under light ether anesthesia, or intramuscularly and subcutaneously without anesthesia, using 0.2 cc of the pH 2 solution containing $4.76 \mu\text{Ci } ^{253}\text{Es}$. One group of

anesthetized rats had 0.15 ml of a pH 2 nitrate solution containing 2 μCi Es applied to 0.786 cm^2 of shaved skin between the shoulder blades for 30 minutes before being gently removed. Groups were housed four rats per cage, and pooled excreta was collected from each cage. Data have been corrected for radioactive decay to the time of administration.

There was translocation of the intravenous Es to skeleton as shown in Figure 1 apparently peaking around day 7 while the early liver burden quite rapidly decreased. All soft tissues appeared to lose about a fourth to a half their 7-day Es burden by day 24 (Table 1) indicating a rapid turnover, much faster than for

Pu. Little difference was seen in the tissue distribution from s.c., i.m., and i.v. administration at 24 days (Figure 1). The actual amount Es retained at 24 days can be approximated by dividing the tissue results in half since the half-life is 21 days. It can readily be appreciated that those tissues showing slow turnover, e.g., bone, are being intensely irradiated.

Only about 8% of the Es remained at the intramuscular injection site by 24 day whereas 16% remained at the subcutaneous site. The Es concentration in the popliteal lymph nodes near the intramuscular injection site was about six times higher than the nodes from the intravenous rats. Similarly,

FIGURE 1. Distribution of ^{253}Es after Administration of $\text{Es}(\text{NO}_3)_3$ to Rats (Corrected for physical decay)

TABLE 1. ^{253}Es Concentration in Tissues After
Administration of $4.76 \mu\text{Ci Es}(\text{NO}_3)_3$ to Rats

		Percent Dose/Gram ($\bar{x} \pm S_{\bar{x}}$)			
Time of Measurement	→	7 Days	24 Days	24 Days	24 Days
Route	→	Intravenous	Intravenous	Intramuscular	Subcutaneous
Tissue					
Femur ^(a)		8.8 ± 0.4	6.8 ± 0.2	5.3 ± 0.5	4.7 ± 0.3
Liver		0.88 ± 0.09	0.16 ± 0.03	.11 ± 0.02	0.11 ± 0.01
Kidney		0.86 ± 0.05	0.33 ± 0.05	0.56 ± 0.08	0.43 ± 0.05
Spleen		0.63 ± 0.01	0.21 ± 0.01	0.13 ± 0.02	0.13 ± 0.04
Lungs		0.12 ± 0.001	0.10 ± 0.01		
Ovaries		0.15 ± 0.02	0.074 ± 0.009	0.062 ± 0.009	0.032 ± 0.006
Heart		0.093 ± 0.002	0.059 ± 0.009		
Muscle		0.012 ± 0.002	.0098 ± 0.0018	0.012 ± 0.004 ^(b)	0.006 ± 0.005 ^(b)
Adrenals		0.12 ± 0.03	0.047 ± 0.008	0.040 ± 0.007	0.040 ± 0.006
Thymus		0.042 ± 0.007	0.029 ± 0.008	0.015 ± 0.003	0.015 ± 0.001
Lymph Nodes:					
Mesenteric		0.12 ± 0.05	0.066 ± 0.005	0.058 ± 0.005	0.029 ± 0.006
Hepatic		0.047 ± 0.008	0.024 ± 0.005		
Splenic		0.12 ± 0.005	0.055 ± 0.012		
Tracheo-bronchial		0.13 ± 0.03	0.027 ± 0.009		
Popliteal			0.061 ± 0.008	0.70 ± 0.18	
Axillary			0.048 ± 0.011		0.088 ± 0.018

a. Based on ash weight. All other tissues based on wet weight.

b. Muscle sampled remote from injection site.

the axillary lymph nodes from the subcutaneously injected rats were slightly higher than the corresponding nodes from intravenously injected rats. Es concentration in the mesenteric lymph nodes for all three routes were similar to other soft tissue results. Generally, tissue concentrations were similar for the three routes of administration with the intravenous route giving slightly higher retention of 24 days.

The patterns of ^{253}Es excretion over the 24-day period for the three routes of administration are shown in Figure 2. The high urinary output of Es after i.m. injection indicates

a very rapid translocation from the injection site; but what is surprising is the anomalous, low urinary output from the i.v.-injected animals on the first day. After about the seventh day, more Es appears in the feces than in the urine; but both are very low and similar in magnitude by the twenty-fourth day. Possibly the liver is the main contributor, other than the injection sites, to the early excretion phase; and, when it is depleted, the excretion rate is slowed to that being released mainly from bone.

Among the bones of the skeleton, Es concentration was greatest in the

FIGURE 2. Excretion of ^{253}Es by Rats After Intravenous, Intramuscular, and Subcutaneous Administration of $\text{Es}(\text{NO}_3)_3$ (Corrected for decay)

vertebrae and sternum (Table 2). The lower concentration in the bones of the feet may be influenced by difficulty in completely removing the surrounding tissue, giving high weights and thus lowering the concentration values; however, these distal bones may have a lower concentration than other bones.

The rats receiving Es solution on the skin absorbed nearly 4% of the total available activity, $2.5 \mu\text{Ci}/\text{cm}^2$, in 30 min. About $10^{-2} \mu\text{Ci}$ were found in the urine, $10^{-1} \mu\text{Ci}$ in feces, 7×10^{-4} , 4×10^{-4} , 2×10^{-4} , and $0.6 \times 10^{-4} \mu\text{Ci}$ on the skin, and in the skeleton, liver, and kidneys, respectively. While the skin site had

TABLE 2. Concentration of ^{253}Es in the Skeleton 24 Days After Intravenous Injection of $4.76 \mu\text{Ci Es}(\text{NO}_3)_3$ at pH 2

Bone	% dose/gram ash weight
	$\bar{x} \pm s\bar{x}$
Femur	6.8 ± 0.2
Sternum	7.8 ± 0.3
Vetebrae	7.9 ± 0.7
Ribs	6.1 ± 0.3
Pelvis	6.6 ± 0.8
Feet	4.3 ± 0.4
Remainder	6.5 ± 0.5

been gently washed to a detectable level of <1-2 cpm using an alpha survey probe, the feces might be high

from additional cleaning of the area by the rat and subsequent swallowing, although the nucchal region would not readily lend itself to such cleaning.

CONCLUSIONS

Generally, Es behaves like a lanthanide and is more mobile and soluble in vivo than Pu. It moved quite rapidly from the intramuscular deposit and from liver but was retained quite tenaciously in bone. The small mass involved may play an important role, as its behavior was reminiscent of low masses of Pm [BNWL-1050, 5.5 (1970)]. Further studies, especially in large animals, would be useful in comparing it with other actinides, in evaluating its hazard, and its behavior towards chelation therapy.

PROMPT AND DELAYED CHELATION THERAPY FOR THE
DECORPORATION OF ^{253}Es FROM RATS USING Zn- AND Ca-DTPA

Investigator:

V. H. Smith

Technical Assistance:

Juanita S. Barnett and K. E. McDonald

When chelation therapy was commenced 7 days after the intravenous administration of $^{253}\text{Es}(\text{NO}_3)_3$, the soft tissue retention was 2 to 5 times greater than when treatment was given after 1 hr. Skeletal retention was doubled from 35% to 77% of that retained by the control rats by delaying the treatment. The relatively short physical half-life and the predominate deposition in skeleton makes prompt treatment imperative if irradiation of the skeleton is to be effectively reduced.

Directly injecting Zn-DTPA near the site of intramuscular deposits of Es reduced local Es retention more than twofold compared to treatments remote from the deposit.

Zn-DTPA appears to be as effective in decorporating Es from rats as Ca-DTPA and, since it is less toxic, is recommended for that purpose.

Since there is a potential for human exposure to Es, its behavior towards chelation therapy, in comparison with other actinides, was evaluated.

OBSERVATIONS

In groups of eight, female Wistar rats, weighing 275 to 300 g were intravenously injected with 4.76 μCi $^{253}\text{Es}(\text{NO}_3)_3$ in 0.20 ml, pH 2 HNO_3 . They were treated intraperitoneally with 1.0 mmol/kg of either the Ca or Zn salt of diethylenetriaminepentaacetic acid (DTPA) 1 hr after the Es administration (prompt treatment). These rats were killed on the seventh

day. Delayed treatment consisted of 1 mmol/kg Zn-DTPA given three times/week for 2 weeks starting on day 7; these animals were killed on the twenty-fourth day. Other rats were injected with 0.10 cc (1.25 μCi) of an $\text{Es}(\text{NO}_3)_3$ solution (pH 2.1) in each rear thigh muscle; a series of Zn-DTPA treatments was started at 1 hr and animals were killed after 24 days. In a similar manner, some rats were injected with Es-DTPA (2.25 μCi) at ratios of Es:DTPA of 2 to 4 and 1000.

Prompt and delayed treatment decreased liver retention markedly as is typically the behavior of soluble, transuranic radionuclides in rat liver (Table 1). Soft tissues and

TABLE 1. Retention of Intravenously Injected $^{253}\text{Es}(\text{NO}_3)_3$ in Rats as Influenced by DTPA Treatments

Tissue	Percent of Corresponding Control Value (Mean $\pm$ 95% confidence limits)	
	Zn or Ca-DTPA Prompt Treatment (a)	Zn-DTPA Delayed Treatment (b)
Liver	6.6 $\pm$ 0.23	13 $\pm$ 7.0
Kidneys	40 $\pm$ 4.8	73 $\pm$ 6.0
Spleen	58 $\pm$ 7.3	49 $\pm$ 5.9
Muscle	30 $\pm$ 2.0	42 $\pm$ 3.1
Ovaries	22 $\pm$ 2.9	100 $\pm$ 7.5
Adrenals	---	57 $\pm$ 9.0
Lungs	---	62 $\pm$ 8.1
Heart	---	108 $\pm$ 16
Mesenteric Lymph Nodes	---	41 $\pm$ 7.3
Femur	34 $\pm$ 4.0	77 $\pm$ 5.6

a. 1 ml physiological saline (controls), 1.0 mmol/kg of Zn- or Ca-DTPA intraperitoneally 1 hr after Es administration. Represents both treated groups of rats since results were not statistically different. Rats killed on 7th day.

b. 1 ml physiological saline (controls) or 1.0 mmol/kg Zn-DTPA given intraperitoneally 7, 9, 11, 14, 16, and 18 days after Es administration. Rats killed on the 24th day.

bone benefited from the early treatment in comparison to delayed treatment. Especially important is the greater effect in the femur since most of the retained Es is in the skeleton. Delayed treatment failed to decrease retention in the ovaries and heart. The latter result is difficult to understand since Es in other muscle was removed by treatment. Only some 20% was removed from the femur with delayed treatment, but the Es appears more mobile than Pu, and continued treatment might well

have removed more Es. The relative ease of decorporation from the liver, spleen, lungs, and lymph nodes suggest that, unlike the situation with Pu, little insoluble particulate Es, resistant to DTPA, is formed on injection of the nitrate.

When Es chelated with DTPA, in 1000-fold excess, was injected intramuscularly in rats and tissues analyzed after 24 days, <0.5% of the initial dose was retained in the rat; but concentrations on a wet weight basis were higher in kidney and spleen (0.015 and 0.013% dose/gram, respectively) than the 0.008% dose/gram found in the femur. This indicates a high stability for the Es/DTPA chelate in vivo, but possibly some breakdown in the kidney, leaving it with a relatively high Es concentration. That body ligands may compete with DTPA for Es was shown by injecting Es-DTPA with what was calculated to be a 2- to 4-fold molar excess of DTPA, which resulted 48 hr later in a body burden of 16% of the initial dose, primarily in the skeleton. Since the masses involved were so small, it is difficult to know if the Es was truly chelated. It would seem prudent, in any case, to use normal doses of DTPA in treating Es incorporation, despite the small mass involved.

For the removal of Es from rats, Zn-DTPA was as effective as Ca-DTPA in the prompt treatment situation (Table 1). The same appears true for Bk. This further extends the usefulness of this less toxic alternative to Ca-DTPA, which had earlier been recommended for delayed therapy and

possibly prompt treatment of incorporation of several actinides [BNWL-280, 81 (1966); BNWL-1550, Part 1, 97 (1971) and A. Seidel and V. Volf. "Removal of Internally-Deposited Trans-uranium Elements by Zn-DTPA," Health Physics, 22, 779, (1972)]. It should be noted that while recommended for the delayed, multiple treatment situation, the Zn chelate was not as effective as the Ca chelate for the prompt treatment of ^{239}Pu deposition in the rat [BNWL-280, 81 (1966)] and is not therefore recommended for prompt treatment in man.

The treatment regimens and results of treating the intramuscularly admin-

istered Es are shown in Table 2. The Zn-DTPA was well tolerated intramuscularly and seemed to be more effective than when the intraperitoneal treatment was used alone. Injecting Zn-DTPA in the vicinity of the intramuscular deposit was beneficial in mobilizing Es from that site.

CONCLUSIONS:

Generally, Es is more susceptible to DTPA therapy than Pu, behaves in this regard like most of the trans-plutonic elements in soft tissue, and is least effectively removed from skeleton.

TABLE 2. Effect of Zn-DTPA on Retention of ^{253}Es 21 Days After the Intramuscular Injection of Both Hind Legs of Rats with 1.25 μCi $^{253}\text{Es}(\text{NO}_3)_3$ in pH 2.1 HNO_3

Treatment	Route	Percent of Corresponding Values from Rats Treated with Physiological Saline(a) (Mean $\pm$ 95% confidence interval about the mean)				
		Liver	Kidneys	Non-treated leg	Treated leg	Femur
Zn-DTPA ^(a)	i.p.&i.m.	3.8 $\pm$ 0.41	44 $\pm$ 4.1	22 $\pm$ 3.9	8.2 $\pm$ 2.4	18 $\pm$ 3.2
Zn-DTPA ^(b)	i.p.	4.3 $\pm$ 0.42	64 $\pm$ 6.2	27 $\pm$ 10	---	42 $\pm$ 7.0

a. 0.10 mmol Zn-DTPA at pH 7.2; 15% given intramuscular at the injection site in one leg (treated leg) + 85% given intraperitoneally at 1 hr, 1, 2, 7, and 15 days, and 100% administered intraperitoneally on 3, 5, 9, 12, and 17 days after the ^{253}Es . 1 ml of physiological saline was administered to the control rats on the same schedule.

b. 0.10 mmol Zn-DTPA given intraperitoneally at 1 hr, 1, 2, 3, 5, 7, 9, 12, 15, and 17 days after the ^{253}Es .

FINE STRUCTURAL EFFECTS OF TEMPERATURE IN FISH

Little is known of the mechanisms of temperature acclimation in fish. This project will examine for immediate ultrastructural changes the organs suspected to be involved - e.g., hypothalamus, lateral line system, swim bladder, and gills - when trout are abruptly transferred from ambient river temperatures to lethal temperatures. Identifying these acute effects should be of assistance in later studies of acclimation to higher but sub-lethal temperatures, particularly with respect to the mechanism of metabolic changes that are known to occur.

PRELIMINARY OBSERVATIONS OF FINE STRUCTURAL
EFFECTS OF TEMPERATURE IN FISH

Investigators:

J. C. Hampton and R. R. Adee

Vascular dilatation, mainly in capillary beds, was consistently found in gills and swim bladders of rainbow trout transferred from water at ambient river temperature to water held at 30 °C. Lateral line organs and the lateral line nerve trunk were also studied in detail in an attempt to resolve some of the uncertainties concerning vacuolization of sensory cells comprising the lateral line organ and separation of myelin layers in the lateral line nerve in fish subjected to temperatures above 25 °C. The results are thus far inconclusive.

Rainbow trout can adapt to temperatures up to about 23-24 °C, some 6 or 7 degrees warmer than that normally found in the Columbia River. Temperatures exceeding 25 °C are lethal. Acclimation to sublethal temperatures is accompanied by compensating shifts in metabolism. These are operative over a range of about 20 °C, far short of the range of about 40 °C which can be accommodated by the Desert Pupfish. In the best interest of proper management of natural resources, this work began as an attempt to examine the reasons for failure of trout to acclimate to temperatures above 25 °C.

Fish were abruptly transferred from tanks at various times during the year, when temperatures were low as 8 °C and as high as 15 °C, to small vessels containing water held at 21-23 °C and at 28-30 °C. Specimens of gills, swim bladder, and lateral line were removed and examined with both the light and electron microscopes.

OBSERVATIONS

No changes were observed in tissues of fish exposed to temperatures of 21-23 °C. However, in each fish exposed to high temperatures, vascular dilatation was clearly evident in gills and swim bladder. The epithelia of these organs showed no change from normal, except for occasional large intercellular spaces at the bases of the cells.

The lateral line organs are spaced about 1 mm apart along the lateral line canal; they consist of sensory hair cells interspersed with supporting cells, some of which secrete mucus. The hair cells are covered by a thick layer of mucus, and they synapse at their bases with branches of the large lateral line nerve. Functionally, the organ has been reported to be sensitive to low frequency vibrations, temperature, atmospheric pressure, orientation in space, and to water currents. Changes in the morphology of the

organ consisted, in general, of vacuolization of both sensory and mucus cells, dissociation of the mucous layer from the surfaces of hair cells, and some disorganization of myelin lamellae in the lateral line nerve and the branches of it which serve each organ. Some of these changes can be observed from time to time in fish killed after exposure to elevated but sublethal temperatures and even at prevailing river temperature to which they were acclimated. Thus, whereas we are reasonably certain that at least some changes in the lateral line organ do occur as a result of exposure to lethal temperatures, more work under more carefully controlled conditions must be done to rule out artifacts attributable to tissue processing.

CONCLUSIONS

The above results have provided some guidelines to the relationship

between elevated temperature and lethality in trout, but the experimental conditions were rather crude in that nothing could be said concerning gas concentrations in a 2.5 gallon vessel of water heated to 30 °C. Work was interrupted when we moved into the new laboratory because we felt that the new facilities with accurate temperature and gas control in large tanks would allow better experimental control. Because of faulty engineering, the facilities have not been available and the fish stock did not survive the move. Temperature control devices are now being installed in two tanks, and a new stock of fish has been ordered. Further experiments will be conducted at carefully controlled temperatures in tanks of suitable size, and the results correlated with gas concentrations prevailing at the time of sacrifice. In this way, we believe more reliable data will be forthcoming.

DEVELOPMENT AND EVALUATION OF BLOOD IRRADIATORS

This project seeks to develop a portable, and possibly implantable, blood irradiator for chronic low-level blood irradiation in patients who would retain full ability to proceed with day-to-day tasks. The irradiator is being developed through the combined efforts of specialists in design and materials, biomedical science, hematology, and dosimetry. Calculated dosimetry parameters provide a framework for testing the effectiveness of individual radionuclide sources in optimizing blood exposure and minimizing radiation to surrounding tissues. The same objectives, plus compatibility at the interface with blood, guide materials selection. Animal testing utilizes beagle dogs with devices implanted in major arteries, comparable in size and blood flow characteristics to sites likely to be selected for human clinical application.

FURTHER DEVELOPMENT OF BLOOD IRRADIATORS

Investigators:

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Technical Assistance:

Linda G. Smith

Following implantation of intracorporeal irradiators, up to 10 rads/day produces no effect on lymphocyte levels in beagle dogs. Formation of ring thrombi at junctions between the artery and the tubular irradiator discouraged further development of tubular irradiators for permanent implantation. Two new approaches are being developed: one is a wire source to be implanted in the center of a vessel; the other is a portable extracorporeal blood irradiator utilizing an external arteriovenous shunt.

Blood irradiation is used to minimize rejection of grafted organs and as a therapeutic measure for some lymphocytic leukemias. Irradiation is now accomplished by utilizing an arteriovenous (A-V) shunt and passing the patient's blood through specially designed gamma or beta irradiators. Because of the heavy shielding required for these irradiators, they are fixed; and the patient must be hospitalized. Inexpensive devices easily carried by the patient would facilitate treatment and extend the clinician's capability to treat more patients. The present effort is directed toward developing such portable devices.

OBSERVATIONS

As reported in the 1970 Annual Report, irradiators utilizing ^{55}Fe or ^{125}I , producing up to 0.5 rad/day to an estimated 940 ml of beagle blood did not produce an observable depression of lymphocyte levels; nor was there any evidence of radiation effects to surrounding tissues when dogs were sacrificed approximately 6 months postimplantation. The only significant surgical sequela was the

consistent formation of ring thrombi at the junction of the dacron prosthetic with the artery. Isotope leakage through the epoxy barrier also occurred.

To gain information about the potential of beta emitters for these irradiators, ^{204}Tl was used. The 0.76 MeV betas from ^{204}Tl dissipate 95% of their energy within the first 0.9 mm of blood. This energy, when used in conjunction with low atomic number irradiator material, reduces the hazard to surrounding tissue from bremsstrahlung radiation.

Four irradiators using ^{204}Tl as the source material were prepared

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according to the basic plan of Figure 1. Two of these were implanted in dogs, but one animal succumbed to embolic obstruction of the anterior mesenteric artery. This was the first instance of early death due to embolism in either the dogs implanted with irradiators or swine receiving basically similar heat exchanger implants. The blood irradiator, though having an adherent thrombus, was not occluded. It is presumed that a thrombus fragment broke loose from the irradiator and lodged in the mesenteric artery. The irradiator in this animal was recovered for more complete dosimetry using the Fricke dosimeter and thermoluminescent dosimeters (TLD). Postimplantation evaluation of dose is not yet completed.

The dog receiving the Mark X irradiator survived and continues in excellent health with no discernible hematologic changes. This irradiator delivers 10.4 rads/day to a presumed

940 ml of dog blood. This is essentially two orders of magnitude higher than the dose reported to produce lymphocyte suppression following whole-body irradiation [L. D. Jacobson, (1954) "The Hematologic Effects of Ionizing Radiation," In: Hollaender (Ed.), Radiation Biology, McGraw-Hill, pp. 1049-54]. There was no evidence of ^{204}Tl leakage into the blood when the epoxy barrier between blood and isotope was supplemented with mylar and silastic.

Two new types of irradiators are now being developed. The first of these is planned to circumvent the problem of ring thrombus constriction of blood flow. This design will not involve replacement of any vessel segment but will consist merely of a relatively fine wire with the center section coated with a thinly encapsulated radiation source. The wire will pass obliquely from one side of the vessel to the other and be sutured

FIGURE 1. Diagram of the Mark X Blood Irradiators Using Beta Radiations from ^{204}Tl

in place at each end. Initial testing will be aimed primarily at determining the likelihood of embolism and movement of the wire.

The second type irradiator is planned for portable extracorporeal use with the A-V shunt already established in patients undergoing dialysis and involved with a kidney transplant operation. The need for such an irradiator has been clearly identified by Dr. D. M. Hume of the Medical College of Virginia (personal communication), and development of this device will be closely coordinated with clinical criteria.

CONCLUSIONS

Up to 10 rads/day to circulating beagle blood does not suppress lymphocyte levels. Persistent occurrence of ring thrombi at the anastomosis of device with blood vessel encouraged us to seek a technique of irradiating the blood without vessel replacement. An implantable wire irradiator is currently under investigation. In addition, an irradiator compatible with the A-V shunts already typically used in patients undergoing dialysis and anticipating a kidney transplant will be developed.

BIOLOGICAL EFFECTS OF INTRACORPOREAL RADIOISOTOPE HEAT SOURCE

This is a new project, first funded during the latter months of fiscal year 1971. Its primary goal is the production of a number of miniature swine preparations in which $^{238}\text{PuO}_2$ heat sources are implanted in the thoracic and/or abdominal cavity for long-term study of the effects of added heat and irradiation. Previous research in our laboratory for the National Heart and Lung Institute has demonstrated the feasibility of implanted electrical heat sources in the descending thoracic aorta of miniature swine. The present project will extend this work to the implantation of radioisotope heat sources.

BIOLOGIC EFFECTS OF INTRACORPOREAL RADIOISOTOPE HEAT SOURCES

Investigators:

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Technical Assistance:

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Thoracic intraaortic implantation of heat exchangers containing 50 watt, medical grade $^{238}\text{PuO}_2$ heat sources for long-term studies of the effects of added endogenous heat and radiation in miniature swine presents a number of bioengineering problems. Through the implantation and 3-month evaluation of three sham devices and by subsequent dress rehearsal implantation of two control exchangers most of these problems have been solved. Five additional heat exchangers are under construction and will be implanted complete with their radioisotope heat sources as soon as those sources are received. Development of techniques for intra-abdominal implantation of such sources is in progress.

Past and current concepts of intra-corporeal cardiac assist and total cardiac replacement systems include the totally implantable power system. One such system under consideration is a heat conversion system powered by a radioisotope heat source. This laboratory is concluding its sixth year of research for the National Heart and Lung Institute (NHLI) on the effects of added endogenous heat in which electrical heat sources were implanted in the descending thoracic aorta of miniature swine for long-term study. This report covers progress to date on a new program, co-

supported by the Division of Biology and Medicine (DBM) and the Division of Applied Technology (DAT-formerly Division of Isotopes Development), on the long-term biologic effects of encapsulated, medical grade $^{238}\text{PuO}_2$ intra-corporeal radioisotope heat sources, generating approximately 50 watts of heat power.

The NHLI-supported study on effects of added endogenous heat has confirmed that, barring greatly elevated ambient temperature or fever, miniature swine weighing 60 kg or more can tolerate indefinitely without detectable physiologic effect 60 watts of added heat via an intrathoracic heat source cooled by slowing aortic blood. A fifty watt power level was chosen for radioisotope heat source studies with this in mind; also, it was felt

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that such a figure meets or exceeds any likely heat dissipation figure for future intracorporeal cardiac assist or replacement systems, representing a waste heat production rate from a system producing 10 watts of usable power at an overall operating efficiency of 20%. Since this efficiency figure is believed to be attainable and since the normal heart performs work at a rate of 10 watts only during transient periods of peak activity, the conclusion is that a 50-watt **source/animal** preparation (operating continuously) provides experimental exaggeration in keeping with standard toxicological procedure.

DAT support was directed toward development and fabrication of the aortic heat exchangers, pertinent dosimetry studies, heat transfer analyses, and theoretical studies of heat pipe concepts which might permit thoracic heat dumping from a source located in the abdominal cavity. DBM support was directed toward surgical studies leading to the production of long-term animal preparations, development of a suitable intraexchanger temperature telemetry system, and intradepartmental engineering support relative to the special problems associated with radioisotope heat source implantation and **bioinstrumentation**.

The NHLI studies, utilizing electrically energized heaters, were perpetually confounded by percutaneous lead breakage, the prime cause of preparation failure. **Radioisotope**-powered heaters eliminate this problem but introduce a few of their own. Discussion of these problems and their

solutions comprise the body of this report.

OBSERVATIONS

The 50-watt $^{238}\text{PuO}_2$ source capsule will be a right circular cylinder 1.28 inches in diameter and 2 inches long, weighing approximately 435 g. Anatomic and thermodynamic considerations resulted in the final aortic heat exchanger design illustrated in Figure 1. Though fine-grained graphite was selected for the body to transfer heat with minimum weight, this exchanger will weigh in the neighborhood of 1.9 lb when charged with one of these sources. The problem of how to securely fix such a massive implant in the dorsal thorax of a quadruped immediately arose, and three sham exchangers were implanted in the descending thoracic aorta of three miniature swine for a 3-month evaluation of fixation techniques. The first idea was completely successful, consisting of a nylon velour fabric flap bonded to the side of the exchanger and delivered through the thoracotomy wound to a subcutaneous position and tacked in place. The flap becomes intimately ingrown with scar tissue and bone from the **periosteum** of the excised rib, rigidly holding the implant in position for however long the fabric may endure in vivo. Monthly radiograms and 3-month postmortem examination of all animals confirmed the success of this method.

Next to percutaneous lead breakage, the most common cause of eventual preparation failure on the NHLI studies was heater occlusion by developing

FIGURE 1. Assembled Heat Exchanger, with Endpieces Coupled but Omitting the Nylon Velour Side Flap on the Body Used to Provide Long-Term in vivo Fixation (The coin is a 25-cent piece.)

thrombi at the junction of aorta and device. This is a common and unsolved problem whenever one attaches a non-porous tube to a blood vessel, and the three sham implants served a second purpose in providing a 3-month evaluation of three methods of aorta-to-device coupling. Early ring-type thrombus formation was found at post-mortem examination in all three cases, and the final endpiece coupling selection was based on two reasons: (1) its thrombi are no worse than in the other two types and (2) the

design permitted rapid establishment of aortic blood flow through the device following its removal from the coolant loop.

The decision was made early in the program to seal the source capsule within the heat exchanger prior to surgery rather than attempt to charge the implanted (empty) exchanger after aortic flow through the device had been established. The latter approach would run the risk of contaminating the exchanger and would be surgically difficult due to the limited space in

which to work following exchanger implantation. Since the graphite lumen of the exchanger must be treated with benzylalkonium chloride and heparin prior to implantation (GBH **antithrombogenic** surface treatment) and since the resulting surface complex is heat labile, the former approach necessitates maintaining the charged exchanger in a coolant loop until just before actual implantation and requires that this implantation be done rapidly so that aortic blood flow may be established before the GBH surface complex is degraded by the rising lumen wall temperature. Theoretical analyses predicted this was feasible; and subsequent bench studies with an instrumented, electrically heated bench model exchanger confirmed that, assuming an initial wall temperature of 4 °C and ordinary ambient temperatures, approximately 5 min are required for the wall temperature to reach the assumed GBH degradation temperature of 66 °C.

A coolant loop was designed and fabricated which circulates refrigerated benzylalkonium chloride solution through the exchanger for at least 12 hr prior to implantation. Approximately 20 min before transfer of the exchanger from the coolant loop to the aorta, valves are switched in the loop which first washes the loop with refrigerated physiological saline and then substitutes refrigerated heparin solution to complete the GBH process. During this time, the exchanger is submerged completely in instrument strength benzylalkonium chloride solution to sterilize the implant. When ready to transfer the exchanger to the animal,

the surgeon lifts the exchanger from the benzylalkonium chloride bath, rinses its exterior with saline, disconnects it from the coolant loop, and implants it between the **endpiece** couplers which have been anastomosed to the cut ends of the aorta. Removing trapped air, aortic blood flow is restored and surgical closure begins.

Dress rehearsals of this procedure were conducted during the preparation of control animals I and II, in which actual heat exchangers (with a lead slug mimicking the plutonium heat source capsule) were implanted in the descending thoracic aorta of two miniature swine (Figure 2) to provide controls for the heated preparation to follow. The first operation prompted a change in the heparin valve-over procedure, and the second prompted a slight change in **endpiece** design to facilitate more rapid implantation. Barring untoward circumstances, exchanger transfer time should never exceed the 5-min limit; even if it should, the exchanger can be returned to the coolant loop for **re-refrigeration** and GBH treatment.

The control II heat exchanger contained a **prototype** temperature telemetry system. By means of a ring counter type of network, consisting of four monostable multivibrators, three having a temperature-dependent **ON** time and the fourth having a fixed (large) **ON** time for identification purposes, three temperatures can be broadcast on a single carrier in the standard **FM** band. Each monostable multivibrator serves to turn the **FM** carrier **ON** or **OFF** in sequence, producing, after limiting and detection,

FIGURE 2. Lateral Radiogram of Control II Immediately Following Implantation Surgery [The base of the exchanger is directed ventrolaterally so that the telemeter circuitry overlies the (mock) source in this view.]

a rectangular waveform in which successive periods are in sequence IDENTIFICATION, T_1 , T_2 , and T_3 . Temperatures T_1 , T_2 , and T_3 refer to the heat source-heat exchanger interface, blood-heat exchanger interface, and body tissue-heat exchanger interface, respectively. The IDENTIFICATION

signal is required by the readout circuitry to identify the subsequent three channels correctly.

The circuit in Control II failed to broadcast soon after removal of the exchanger from the coolant loop. Subsequent reruns with the bench model heat exchanger revealed that the large

temperature excursions involved could interrupt the ring counter and, once implanted in the animal, the counter could not be retriggered into action. The circuit is presently being modified, replacing the IDENTIFICATION monostable multivibrator with an astable multivibrator to eliminate this problem.

Animals Control I and Control II continue on experiment at present, apparently in fine health. Upon receipt of the plutonium heat source capsules, implantation of thoracic heat exchangers containing those capsules will proceed. Five exchangers are under construction for that purpose. No particular difficulty in implantation is anticipated; however, the postoperative period may introduce such problems as hyperthermia during anesthetic recovery, late hyperthermic episodes and/or augmentation of pyrexia states, and partial to complete occlusion of blood flow through the exchanger by ring thrombus development at the ends of its lumen. Barring greatly elevated ambient temperature (in excess of 85 °F) or severe pyrexia states, hyperthermia should be manageable by cold showers provided for this purpose. The problem

of ring thrombus formation is currently beyond our control; and, until some solution to this phenomenon is found, we have no choice but to hope for early thrombus stabilization, as seen in some of the past NHLI electrical heater preparations.

Abdominal implantation of source-bearing heat exchangers, cooled by flowing aortic blood, is a difficult problem that will be explored in two ways. The first consists of rerouting thoracic aorta flow through an arterial prosthesis to the terminal aorta, resulting in retrograde abdominal aorta flow from the postrenal aorta cranially, and interposing the heat exchanger in this prosthesis at a later date (when patency of the bypass is confirmed). The second approach involves the engineering development of a suitable heat pipe which will carry heat from a heat source in the caudal abdomen to a heat exchanger in the thoracic aorta. The engineering design problems of the latter approach are very difficult in themselves, and it has been tentatively decided to begin with implantation of a suitably similar sham heat pipe to test for long-term functional compatibility.

1971

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