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SOME EFFECTS OF Co^{60} GAMMA RADIATION ON CHICK EMBRYOS

Darryl L. Hessel* and S. Phyllis Stearner

In 1904, Gilman and Baetjer,⁽¹⁾ experimenting with the effects of the newly discovered Roentgen rays on chick embryos, noted hemorrhages, retarded development, various deformities and patchy feather patterns. In the last half century, experiments with X and γ rays have yielded similar results and, in addition, have shown some effects on survival. (2-6)

The purpose of this investigation was 1) to describe the effects of Co^{60} γ radiation on the gross morphology of chick embryos of various ages, and 2) to determine the sensitivity of these embryos to doses of Co^{60} γ radiation, as measured by 24-hr mortality.

Materials and Methods

The embryos used in this study were of white leghorn stock obtained from Creighton Brothers, Warsaw, Indiana. Eggs were received at the laboratory within 1 day after shipment from the hatchery. Upon arrival they were either placed immediately in an incubator or stored at room temperature until needed. The storage period was never longer than 7 days. Storage of more than 5 days resulted in considerable early natural mortality of the embryos and a higher percentage of apparent infertility. Incubation was carried out in a Jamesway incubator maintained at a constant 63% relative humidity and 36°C temperature.

Several hours prior to irradiation all eggs to be exposed at incubation ages of 3 days or more were candled for fertility and survival. The presence of a visible extraembryonic circulatory system and, in some cases, movement of the embryo itself, were taken as evidence of life.

Irradiation was carried out in the high-level γ -ray irradiation facility. The rates of exposure were controlled by varying the distances between the eggs and the 5000-curie Co^{60} source. The eggs were maintained in position during irradiation by packing them into half-dozen-egg cartons, which were then placed on end on shelves surrounding the source. The temperature of the eggs was maintained near 36°C by warming the eggs in the incubator after packing them and keeping them in warmed insulated bags when out of the incubator, except during the actual period of irradiation. The length of exposure was 10 min, except for total doses higher than 1700 r, which required 15 min.

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Examination of the eggs at regular intervals after irradiation was carried out by candling or by serial sacrifice. When embryos were to be followed for day-by-day survival, the eggs were candled each day. Eggs containing dead embryos were opened and the gross morphology of the embryos was examined. In all embryos irradiated at incubation ages of 3 days or more, cessation of the heart beat was taken as the final criterion of death.

In embryos irradiated after only 2 days of incubation, whitening of the tissues and a weak or irregular heart beat were taken together as the criterion of death. Since cardiac muscle is extremely resistant to irradiation damage, the heart may go on beating long after circulation has ceased and other tissues are dead. When the objective of an experiment was description of progressive post-irradiation changes in morphology, or when embryos were too young to be seen by candling, the eggs were opened and the embryos examined.

Eggs incubated 3 days or less were opened into a bowl of 123 mM saline solution.⁽⁷⁾ The embryos and their intact extraembryonic systems were then cut free of the vitelline membrane, floated onto a watch glass, and examined under a dissecting microscope. Older embryos were examined with the naked eye.

The study of radiation mortality involved 507 controls and 3108 irradiated embryos; points on the graphs represent 20 to 60 individuals. From these points the $LD_{50}/24$ -hr values were estimated through the use of the probit transformation. Least squares lines for the probit points were calculated on an IBM 1620 computer. Data from 286 additional controls and 1526 additional irradiated embryos were included in the study of gross morphological changes due to radiation damage. Included in the latter number are all embryos irradiated at 24 or 36 hr of incubation.

Results

Mortality. The LD_{50} 's estimated from the 24-hr postirradiation mortality data (Figures 1 and 2) present an interesting pattern when plotted against preirradiation incubation age (Figure 3). Radiosensitivity increases sharply between 2 days (LD_{50} of 1375 r, with a correction from observed value estimated on the basis of general shift in the rate of development of eggs as described in the discussion) and 9 days (LD_{50} of 708 r). It then falls off gradually to 15 days and rises again in the 18-day embryos. The radiosensitivity of 3- to 4-day hatched chicks exposed at rates similar to those used in this study has been shown to be lower than that of 15-day embryos.⁽⁸⁾

Figure 1
Dose-mortality relations
(probit transforms) for
embryos of various ages,
as measured by death
within 24 hr (data from
September 1962 to
January 1963).

Figure 2
Dose-mortality relation (probit transforms) for
2- and 3-day embryos, as measured by death
within 24 hr. Data from August-September 1963
are compared with those from September 1962 to
January 1963.

Figure 3
The 24-hr LD₅₀ for embryos of various ages

Probability of death at 1 day, for embryos of various ages, is not correlated with probability of death on the second and third days. For example, in each of 3 groups of embryos, irradiated at incubation ages of 3, 4, and 15 days, 80% were dead within 3 days after exposure (Figure 4). While in the 15- and 4-day groups the greatest number of embryos died during the first day (79% and 57% respectively), only 2% of the 3-day group died in that period. The response in the 4- and 15-day groups is typical of all groups irradiated after 4 or more days of incubation.

Figure 4

Mortality 24 hr after irradiation in groups showing equivalent (80%) total 3-day mortality

General description. Death occurring during the first day after irradiation was characterized by a general circulatory breakdown. Embryos irradiated at or before 36 hr of incubation developed no circulation beyond the blood-island stage. Growth and development of the embryo itself was apparently stopped or severely inhibited. In some eggs opened only a few hours after irradiation, the embryo itself was not distinguishable. Only the blood islands were visible. With doses at which some development occurred there was usually no evidence of torsion (compare Figures 5a and b).

In a detailed investigation carried out on embryos after 2 and 3 days of incubation, eggs were opened at 2, 4, and 6 hr, and 1 day after irradiation. This study revealed that definite signs of circulatory breakdown are evident in these embryos 2 to 3 hr after irradiation. At this time there was more or less complete stasis in the sinus terminalis and many of the small vessels of the extraembryonic circulatory system. The embryos irradiated after 3 days of incubation also displayed superficial hemorrhages in the head and intermyotome areas. Plugging of the intraembryonic vessels and of the large extraembryonic vessels was also evident in many embryos of both age groups at this time.

Six hours after irradiation, the circulatory breakdown in the 2- and 3-day embryos was more complete. Especially notable was the frequent occurrence of massive hemorrhages into the chorionic sac. By this time other symptoms of irradiational damage were evident. General edema and clouded, non-viable tissues were the most obvious of these.

Figure 5. A, control embryo, 48-hr incubation; B, embryo exposed to 1500 r in 15 min at 24-hr incubation, observed 24 hr later; C, control embryo, 54-hr incubation; D, embryo exposed to 1500 r at 48-hr incubation, observed 5 hr later; note severe intermyotome hemorrhages (arrows). A and B were from the September 1962 to January 1963 series; C and D were from the August to September 1963 series.

Embryos irradiated at 2 days of incubation and examined 1 day later displayed severe signs of irradiation damage. In many the heartbeat was stopped or weak and irregular. Circulatory difficulties evident earlier were more severe and more generally present. Intraembryonic vessels were congested with blood clots, both circulating and adhering to the endothelium. Superficial hemorrhages were present in nearly all the irradiated embryos. Edema, whitening of the tissues, and underdeveloped eye vesicles were common.

The embryos irradiated at 3 days of age displayed one day afterwards about the same symptoms as did the 2-day embryos. In addition they also exhibited massive hemorrhages into the brain cavities. The heartbeat was absent in many of them.

During examination of the 2- and 3-day embryos, a record was made of stage of development, based on the series described by Hamburger and Hamilton.⁽⁹⁾ It was found that rate of development was severely decreased in irradiated individuals in proportion to the amount of radiation received (Figure 6).

Death within 1 day after irradiation was characterized in older embryos by severe circulatory breakdown. Those embryos irradiated after 4 days of incubation typically displayed blood-engorged brain cavities (Figure 7) and allantoic sacs, and severe hemorrhages of the eyes, the limbs, and the intermyotome spaces. The heart and large blood vessels were often severely congested. Embryos of 15 or 18 days of incubation at the time of irradiation displayed severe hemorrhages of the kidneys, liver, brain, cardiac muscle, and occasionally lungs. Their entire bodies were hyperemic. Subcutaneous edema and ascites were observed in some individuals. When feathers were present they were often only loosely anchored to the skin. Embryos irradiated at 9 days of incubation usually had badly malformed mandibles.

Figure 7

A, control embryo, 4 days' incubation; B, embryo exposed to 1100 r in 10 min at 3 days' incubation, observed 24 hr after exposure. Note general edema, severe hemorrhages in the mesencephalon, metencephalon, and myelencephalon, in the optic vesicle and intermyotome areas.

Embryos of all ages that died during the period from 2 to 5 days after irradiation showed inhibited growth and development and severe edema of body tissues and extraembryonic membranes. The nervous system of those individuals irradiated after only 36 hr of incubation showed a general swelling of all parts. The same pressure in the nervous system of the 2-day embryos often caused a conspicuous swelling of the thin-roofed myelencephalon. Eye vesicles in these very young embryos were found to be poorly developed. Anencephalia, often found in other vertebrate embryos irradiated during gastrulation,⁽¹⁰⁾ was common among chick embryos irradiated at 36 hr of incubation or before.

The only embryos observed more than 5 days after irradiation were those exposed at 3 or 4 days of incubation. At hatching time these individuals showed severe edema, dwarfing, and severe malformation of the limbs and, in some cases, of the mandible (Figure 8). It was often noted that the left lobe of the liver was much smaller than the right.

Discussion

Most of the work involving embryos irradiated at 2 and 3 days of incubation was carried out during August and early September, 1963. The other work was completed in January, 1963. Comparison of the radio-sensitivity of embryos in the 2 periods revealed that those used in the later period were generally more sensitive than those used earlier (Figure 2). The LD_{50} shown in Figure 1 for 3 day embryos has, therefore been corrected for better comparison with the values from the earlier series.

Figure 8. A, chick embryos exposed at 4 days' incubation, observed 16 days after exposure (20 days' incubation). Note increasing severity of dwarfing effect as dose increases. 1, control; 2, 800 r; 3, 900 r; 4, 1000 r. B, comparison of posterior limbs of control chick embryo incubated 18 days (second and fourth from left) and embryo exposed to 1000 r in 10 min after 3 days' incubation, observed 15 days after exposure (first and third from left). Note difference in size and general appearance.

The difference in sensitivity of the eggs used in the two periods is probably attributable to differences in the stage of development reached after 48 hr of incubation. Forty-eight-hr controls between September, 1962, and January, 1963, were developed to stages 12-14, as defined by Hamburger and Hamilton,⁽⁹⁾ while after the same length of incubation, the controls of the August and September, 1963 period were developed to stages 14-18. Since sensitivity in the young embryos increases with age, the general increase in sensitivity in the second of the two periods may be due to the increase in the developmental age.

The increasing radiosensitivity of the chick embryo as it progresses from 3 to 9 days of incubation suggests a correlation between this sensitivity and the increasing size and complexity of the organism. The fact that circulatory breakdown precedes death in these embryos supports this idea. Apparently the tissues of the older, larger individuals are more dependent upon the circulating blood for obtaining nutrients, disposing of wastes, and carrying on gaseous exchange.

Support for this view comes from several sources. First, comparison of the 3 groups of embryos irradiated at 3 ages and followed for 3 days revealed that although total 3-day mortality was the same in all of the groups, the older embryos died earlier than younger ones (Figure 4). Second, the work of Strangeways and Fell⁽²⁾ and Schneller⁽³⁾ demonstrated that death of the tissues in irradiated chick embryos was due, not to direct radiation damage to the cells, but to changes in the environment of the cells. Finally, the number of embryos showing early damage (within 2-3 hr) to the extraembryonic circulatory system in groups given various doses of radiation after 2 and 3 days of incubation has proven to be a relatively accurate prediction of the number of embryos in each group which will be dead one day after irradiation. The circulatory breakdown may thus be considered a logical cause for death.

We have no satisfactory explanation for the gradual decrease in radiosensitivity of the embryos between 9 and 15 days of incubation, but the increase at 18 days is undoubtedly due to the period of high natural mortality associated with hatching.

Early observation of embryos irradiated at 2 and 3 days respectively has shown that clots develop in the blood vessels and that the blood becomes generally more viscous. Massive hemorrhages into the extraembryonic cavities and into the brain areas are evidence of damaged vessel walls. These factors in combination reasonably explain the circulatory breakdown so evident in all the irradiated embryos. The observed edema indicates that some of the blood fluids have escaped from the vessels into the interstitial areas of the tissues and suggests that the osmotic balance between the blood and the tissues has been disturbed.

Anencephalia, poor eye development, and malformed limbs are evidence that those cells most actively dividing at the time of irradiation are most severely damaged. In the embryo of 36-hr incubation, the most actively dividing cells are those in the tissues of the brain. Later, cells in the eye vesicles and in the limb buds are more active.

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FRACTIONATED IRRADIATION OF MICE WITH FISSION NEUTRONS AND Co⁶⁰ GAMMA RAYS

Howard H. Vogel, Jr., and Donn L. Jordan

There is evidence, ⁽¹⁾ confirmed in several laboratories, that the relative biological effectiveness (RBE) of neutrons over gamma rays is increased from approximately 2 or 3 for single acute exposures to as high as 5 to 15 for irradiation with small doses over long periods of time. ⁽²⁾ The experiments reported here were planned to answer certain questions which would probably be helpful in the experimental design of the JANUS program:

1. What is the relative biological effectiveness of fission neutrons and Co⁶⁰ gamma-rays when exposures are carried out over a protracted period of 3 months?
2. Does this RBE figure resemble that for single acute exposure or the increased figure for length-of-life irradiation?
3. What effect does fractionation of the neutron dose have on mean survival time?
4. What is the comparative effect of these two radiations on the incidence of thymic tumors following fractionated exposures?

Experimental Plan

Female CF No. 1 mice, 9-10 weeks old, were exposed to either fission neutrons or Co⁶⁰ γ -rays in the gamma-neutron radiation chamber ⁽³⁾ at CP-5. The experimental plan is shown in Table 1. The dose rate was approximately 0.75 rads/min for the neutrons and 0.25 rads/min for the gamma rays. In this experiment the doses were based on an assumed RBE (n/γ) of approximately 3 since previous fractionation experiments had indicated clearly that an RBE (n/γ) of 5 was too high. ⁽⁴⁾

TABLE 1
Experimental plan

Radiation	Number of mice exposed	Fractionation, exposures per week	Dose per exposure, rads	Total dose in 13 weeks, rads
Fission Neutrons	32	1	60	780
	32	3	20	780
	32	6	10	780
Co ⁶⁰ γ -rays	24	1	180	2340
	24	3	60	2340
	24	6	30	2340

Results

The experiment is not fully completed since some of the irradiated animals are still living. However, certain trends are already clearly indicated, and we are presenting this paper as a progress report 260 days after the first irradiations. Statistical analysis of the final data will be carried out when all the mice have died.

Since the three sub-groups for each radiation showed similar mean survival times, the data were pooled, and the cumulative mortalities for the two radiations were compared (Figure 9). It is clear from these results that 50% of the mice irradiated with either gamma rays or neutrons were dead by approximately 200 days after the first exposure. Although there is some evidence that the neutron-irradiated animals are dying at a slightly faster rate than the γ -irradiated ones, the cumulative mortality curves are very similar throughout most of the postirradiation period, with a maximum difference of only about 10% mortality. It is evident that the dose ratio of approximately 3 used in this experiment produces very similar mortality patterns in the two irradiated groups. Under the conditions of exposure at the CP-5 reactor, fission neutrons have been found to be almost three times as effective as Co^{60} γ -rays for 30-day lethality following single doses of the two radiations.⁽⁵⁾ Thus, the first general conclusion of this experiment is that protracting the neutron or gamma-ray exposure over a 13-week period does not appear to raise the n/γ RBE significantly above the figure already reported for single brief exposures.

Figure 9

A comparison of cumulative mortality in two groups of mice irradiated over a 13-week period. Group 1 was exposed to 60 rads of fission neutrons per week and group 2 was irradiated with 180 rads of Co^{60} γ -rays per week. During this 260-day period, 3/56 (5%) of the unirradiated control mice had died.

This conclusion is strengthened by an experiment reported earlier.⁽⁴⁾ Its plan was essentially the same as the replicate reported here, except that an RBE of 5 rather than 3 was selected as the basis for the dose ratio. Mice were exposed to the same total weekly neutron dose of 60 rads, delivered in the same three fractionation patterns, but the mice exposed to Co^{60} γ -rays received 300 rads a week instead of 180 rads. The mean survival time of the γ -irradiated mice was less than 100 days, and it was clear that the figure of 5 was too high for the neutron/gamma RBE under the conditions of this earlier experiment.

Fractionation of the Neutron Dose

Figure 10 compares the effects on cumulative mortality of fractionation of the neutron dose into one, three, or six exposures each week. This graph gives the cumulative mortality curves for these three groups of irradiated mice for the earlier and the present neutron experiments combined. The replicate neutron experiments were carried out at slightly different dose rates, the earlier one at approximately 1.1 rad/min and the present experiment at 0.75 rad/min. There were at least 60 mice in each fractionation group.

Figure 10

A comparison of cumulative mortalities in three groups of neutron-irradiated mice. Each group was irradiated with 60 rads of fission neutrons per week for 13 weeks at a dose rate of 0.75-1.1 rad/min. The groups received their weekly exposures in one, three, or six fractions.

When mice are irradiated with 60 rads of fission neutrons each week for a 13-week period of exposures, fractionation of the dose into 1, 3, or 6 parts per week does not seem to change the mortality pattern significantly. The slopes of the three lines are similar and autopsies reveal the same pathology in each group. All three subgroups showed a median survival time of approximately 190 days.

In Table 2 we have listed the mean survival times of the decedents. Since there are a few survivors in each group, the final mean survival times will be elevated somewhat. It seems improbable that there will be any significant difference between the survival times of the mice exposed three or six times a week. There is an indication that the animals exposed once a week may live slightly longer than the other two groups, but the significance of this difference will have to await final analysis.

The tentative conclusion is that fractionation of the weekly neutron dose, under these experimental conditions, into 1, 3, or 6 fractions has no significant effect on the mean survival times of the irradiated mice.

TABLE 2

Mean after-survival times of mice exposed to 60 rads of fission neutrons each week for 13 weeks

Neutron fractionation group	Mean after-survival time, days*	Number of mice dead	Number of mice still alive (8/15/63)
6 x/week	183.1	56	7
3 x/week	183.8	57	3
1 x/week	191.6	55	9

*These figures include all mice from the first neutron experiment and all decedents (up to 260 days) from the present experiment.

Comparative Incidence of Thymic Tumors

After the mice had been irradiated over the 13-week period, they were retained in our animal rooms for the length of their lives. Death checks were made twice daily. Every mouse in the experiment was autopsied. It soon became evident that thymic tumors were observed in almost every mouse that died following these fractionated exposures to neutrons or γ rays from Co^{60} .

Seventy-six neutron-irradiated mice have died in the three fractionation groups between 114 and 258 days after the start of irradiation. Of this group, 68 (90%) showed evidence of a reticular tumor of the thymus gland at autopsy. The tumor usually completely surrounded the heart. Spleen, liver, and lymph nodes were usually, but not always, enlarged. In the γ -irradiated groups, during the period up to 260 days, 41 of 47 mice (87%) showed this thymic tumor. It is probable that these very high tumor percentages may reach close to 100% during this period of high mortality since our figures of approximately 90% are based on observations at autopsy without further histological study.

There does not seem to be any significant difference in the incidence of these tumors between the neutron and the γ -irradiated mice. The same high incidence of the tumors was observed whether the weekly dose of either radiation was fractionated into 1, 3, or 6 exposures. Evidently, 13 weekly doses of 60 rads of neutrons or 180 rads of γ -rays will produce this tumor in at least 9 out of 10 of the exposed mice within the 6-month postirradiation period.

A few other tumors were observed at autopsy. Those observed in the neutron-irradiated mice were two mammary tumors, one ovarian tumor, one tumor involving a muscle of the leg, and one tumor of the intestine. A tumor of the rectum was also noted in one γ -irradiated animal.

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PARAMETERS OF RADIATION DOSAGE THAT INFLUENCE THE PRODUCTION OF OSTEOGENIC SARCOMAS IN MICE

Miriam P. Finkel, Patricia B. Jinkins, and Birute O. Biskis

The carcinogenicity of radionuclides that may gain entrance into the body is related to certain of their physical and chemical properties. In the production of bone cancer by radioactive materials that localize in the skeleton, α -emitters are more effective than β -emitters, strong β -rays are more effective than weak β -rays, and isotopes with half-lives measured in weeks, months, or years are more effective than isotopes with very short half-lives. The recognition of these relationships and of others associated with the daughter products of radioactive decay has made it possible to predict the carcinogenicity of untested bone-seeking isotopes under a set of standard conditions. The standard test conditions include the administration of the radionuclide in a single intravenous injection to 70 day-old CF No. 1 female mice.

With this information as a foundation, we are now studying the production of osteogenic sarcomas in mice when the standard conditions are changed. In the series of experiments described here, we gave particular attention to the association between several factors of radiation dosage and the carcinogenic response. The variables under investigation were intensity, duration, and magnitude of exposure. The five major experiments contributing to this report are at several stages of completion. All the data are available in one group, only the histopathologic study remains to be done in two, but a large proportion of the animals are still alive in the remaining two. The reliability of the comparisons and the soundness of the interpretations that can be made at this time depend, of course, upon the degree of completeness of the studies. However, except for the latter two, the trends of the results are already firmly established, and adding or subtracting a few osteogenic sarcomas as the data become complete will not change the general picture substantially.

The experiments will be discussed under three main headings: fractional exposure, continuous exposure, and time-limited irradiation. Fractional exposure was accomplished by administering the total amount of Sr^{90} or Ca^{45} in 1, 5, or 20 injections. This procedure changed the temporal and spatial pattern of absorbed dose without changing the total dose received by the mouse. Continuous exposure was accomplished by maintaining mice as long as they lived on food with a constant Sr^{90} -to-calcium ratio. This procedure provided a gradual accumulation of Sr^{90} in the body, and it assured a more uniform spatial distribution of the dose. Time-limited irradiation was accomplished by using as the carcinogenic agent either Y^{90} , which has a physical half-life of 64 hours, or X-rays. These sources permit manipulations of dose rate and duration that are not possible when longer-lived radionuclides are administered.

1. Fractional Exposure

In the fractional exposure experiments Sr^{90} and Ca^{45} were injected intravenously, the total amount being given either in a single injection, in 5 injections once a week for 5 weeks, or in 20 injections 5 times a week for 4 weeks. Three levels of each nuclide were used: 1.0, 0.5, and 0.25 μc Sr^{90} /g body weight (in equilibrium with Y^{90}), and 6.0, 4.0, and 2.0 μc Ca^{45} /g body weight. There were 510 Sr^{90} -injected mice, 300 Ca^{45} -injected mice, and 135 controls that provided information on osteogenic sarcomas. Additional animals were used for autoradiography, whole-body counting, and radioanalysis.

Results

Body burden. The amount of Sr^{90} and Ca^{45} in the body under the three regimens of injection is shown in Figure 11. Initially there was a substantial difference in body burden depending upon number of injections, but the difference had essentially disappeared by 20 days. After the last injection, which was on day 28 for 5 fractions and day 25 for 20 fractions, the animals that had received multiple injections contained more of the radioisotope than those that had received a single injection. This difference became smaller with increasing time.

Figure 11. Body burden of Sr^{90} and of Ca^{45} as a function of time after intravenous administration. The total amount was given in 1 injection, in 5 injections at the rate of 1 a week, or in 20 injections at the rate of 5 a week. The shaded area includes the minimum and maximum when 20 injections are given.

The retention of Ca^{45} was much higher than the retention of Sr^{90} , and, were it not for the fact that the half-life of Ca^{45} is only 165 days, the dissimilarity between the 2 curves would have been even larger. Because

of unequal retention, comparisons of body burdens of Sr^{90} and Ca^{45} when they are given in 1, 5, or 20 injections show somewhat different relationships. For example, the maximum Sr^{90} burden after 5 injections was 48% of the maximum after a single injection whereas the comparable value for Ca^{45} was 74%.

Autoradiography. The three injection schedules produced different patterns of deposition of the radionuclides. Autoradiographs of femurs of mice that had received Sr^{90} appear in Figure 12. These pictures were prepared from thin bone sections, between 50 and 80 μ thick, so that autographic localization would be distinct.

Points of special note are the following: 1) The deposition of Sr^{90} was very heavy in the distal epiphyseal plate and adjacent metaphysis during the first week after injection because the bone was still growing. 2) Sixty-three days after the injection of 0.5 $\mu\text{c/g}$ the metaphyseal strontium had disappeared. 3) Even 100 days after the injection of 1.0 $\mu\text{c/g}$ a heavy deposit remained in the metaphysis. Vaughan and Owen have reported a similar failure of resorption of Sr^{90} in the rabbit tibia after the injection of 0.6 $\mu\text{c/g}$.⁽¹⁾ This particular femur is 2% longer than those taken 5-8 days after injection, but it is 2.4% shorter than the other 100-day specimens. Possibly the retention of metaphyseal strontium was associated with decreased growth. 4) A band of autoradiographic darkening appeared in the metaphysis at 63 days when 0.5 $\mu\text{c/g}$ had been given in 5 or 20 fractional injections over a period of 4 weeks. 5) In general, the autoradiographic appearance was very similar whether 0.5 $\mu\text{c/g}$ had been given in 5 fractions or in 20.

Autoradiographs of femurs of mice that had received Ca^{45} appear in Figure 13. Specimens from mice that had received 6.0 $\mu\text{c/g}$ were selected for autography for practical reasons based upon the half-life of Ca^{45} . However, had we known that the carcinogenic response would be greater after the injection of 4.0 $\mu\text{c/g}$, we would have used that level. The possibility exists that the smaller response at the higher level is associated with a metabolic disturbance of the bone that could be reflected in the autoradiographic picture. However, in a previous study⁽²⁾ the proportion of injection Ca^{45} measured autoradiographically in mouse femurs was independent of the amount administered over a range of 0.25 to 6.0 $\mu\text{c/g}$.

The following features of Figure 13 are of particular interest. 1) The localization of Ca^{45} 4 hr after injection was similar to the early localization of Sr^{90} . 2) Twenty-eight days after injection the distal metaphysis did not appear on the autoradiograph, and the distal epiphyseal plate was much less intense than the cortex. 3) The autoradiographic appearance of the femur 58 days after injection was the same as it had been at 28 days. 4) Four hours after the last of 5 or 20 fractional injections the darkening of the epiphyseal plate was very dense. 5) At 58 days the darkening of the

Figure 12. Auroradiographs of thin sections of femurs arranged according to the amount of Sr^{90} injected, the number of fractional injections, and the time after the first injection

$6\mu\text{c Ca}^{45}/\text{gm}$

Figure 13. Autoradiographs of thick sections of femurs arranged according to the number of fractional injections of Ca^{45} and time after injection. The total amount was $6\mu\text{c/g}$.

epiphyseal plate was still very dense if 5 fractional injections had been given. However, autoradiographic darkening was much less intense if 20 fractional injections or a single injection had been given.

Life span. Life expectancy of each group of isotope-treated animals was less than the life expectancy of the controls (Figure 14). Except for $1.0 \mu\text{c Sr}^{90}/\text{g}$, it made little difference into how many fractions the total amount had been divided. Life span decreased as the amount of Sr^{90} increased, but $4.0 \mu\text{c Ca}^{45}/\text{g}$ had as great an effect as $6.0 \mu\text{c/g}$.

Figure 14. Average number of days lived after the first injection as a function of the total amount and the number of injections

Osteogenic sarcomas. The incidence of osteogenic sarcoma and the expectancy of death with these neoplasms are presented in Figure 15. The 600 Sr^{90} mice had a total of 1119 malignant bone tumors, and the 345 Ca^{45} mice had 525. Tumor incidence, which was calculated by dividing the total number of tumors by the total number of mice, is the customary value, but tumor expectancy provides a more reliable index of carcinogenicity because it is not as seriously influenced by incidental deaths that may reduce a population quite early. Tumor expectancy is comparable to life expectancy, and it is calculated by dividing the number of tumors still to appear by the number of animals still alive. Since tumor expectancy remained relatively constant from 150 days after injection until less than 3 animals remained alive, the average of the values obtained at 10-day intervals was used. The advantage of tumor expectancy over tumor incidence is well illustrated by these two experiments. In the Sr^{90} experiment the relationships among the groups are not the same when tumor incidence and tumor expectancy are considered. In the Ca^{45} experiment, on the other hand,

there is very little difference. Both of these experiments were relatively free of infectious disease. The "incidental deaths" that reduced the population in the Sr^{90} experiment and distorted tumor incidence were deaths due to lymphocytic neoplasms.⁽³⁾ These tumors were not increased in the Ca^{45} experiment.

Figure 15. The incidence and expectancy of osteogenic sarcoma as a function of the total amount and the number of injections. Tumor incidence: total number of tumors per total number of mice.

Tumor expectancy: number of tumors to come per number of living mice, calculated at 10-day intervals and averaged from 150 days after injection until less than 3 animals remained alive.

Tumor expectancy after Sr^{90} administration decreased as the total amount injected decreased. It also decreased as the number of fractional

injections increased. The same general relationships appear in Figure 16, where the rate of mortality with osteogenic sarcomas is plotted. The daily disease-specific mortality rate was derived from the rate calculated every 10 days for a 50-day period from the time of injection until less than 3 mice remained alive.

Figure 16. Daily rate of death with osteogenic sarcoma as a function of time after the first injection of Sr^{90} . A total dose of 1.0, 0.5, or 0.25 $\mu\text{c/g}$ was given in 1, 5, or 20 injections.

Tumor expectancy after Ca^{45} administration did not follow a simple pattern (Figure 15). The carcinogenicity of 6.0 $\mu\text{c/g}$ was no greater than that of 4.0 $\mu\text{c/g}$, and at each dosage level 5 injections resulted in more osteogenic sarcomas than either a single injection or 20. The disease-specific mortality rates in Figure 17 show that 6.0 $\mu\text{c/g}$ actually was less carcinogenic than 4.0 $\mu\text{c/g}$. Therefore, the data at the highest level are not useful for evaluating the effect of fractionation. When the total dose was 4.0 or 2.0 $\mu\text{c/g}$, a single injection was considerably less effective than multiple injections, and 20 were less effective than 5.

Tumor expectancies are compared in another fashion in Figure 18. Besides emphasizing the decrease in carcinogenicity in going from 1 to 5 to 20 fractions of Sr^{90} and from 5 to 20 to 1 fraction of Ca^{45} , the non-linearity of the dose-response curve is illustrated. There was one osteogenic sarcoma among the Ca^{45} control mice, giving a tumor expectancy of 0.03. An extension of the line connecting 2 and 4 $\mu\text{c Ca}^{45}/\text{g}$ injected in 5 fractions comes reasonably close to that value. However, extensions of

each of the other lines transect the abscissa at values greater than zero. Reduction in dose level from 0.5 to 0.25 $\mu\text{c}/\text{Sr}^{90}/\text{g}$ resulted in an average reduction in tumor expectancy to 1/5. Reduction in dose from 4.0 to 2.0 $\mu\text{c}/\text{Ca}^{45}/\text{g}$ resulted in an average reduction in tumor expectancy to 1/2.3.

Figure 17. Daily rate of death with osteogenic sarcoma as a function of time after the first injection of Ca^{45} . A total dose of 6.0, 4.0, or 2.0 $\mu\text{c}/\text{g}$ was given in 1, 5, or 20 injections.

Figure 18. Average expectancy of death with osteogenic sarcoma after 150 days as a function of amount injected. The Ca^{45} scale of $\mu\text{c}/\text{g}$ is 5 times higher than the Sr^{90} scale.

Discussion

Although the carcinogenicity of fractional exposure to Sr^{89} and Ca^{45} has been reported for rats,^(4,5) the only study, apart from those presented here, comparing the response to a single injection and to multiple injections of the same total amount of a radionuclide is that with P^{32} reported by Blackett⁽⁶⁾ and Lamerton.⁽⁷⁾ They found that 3 $\mu\text{c/g}$ given in 1 injection produced 3 bone tumors in 20 rats, whereas when 1 $\mu\text{c/g}$ was followed at 2-week intervals by 4 injections of 0.5 $\mu\text{c/g}$, there were 19 tumors in 20 rats. This increase in response with fractionation was even more marked than the increase we observed with fractionation of Ca^{45} in mice. We obtained contrary results, however, with single and multiple injections of Sr^{90} .

The Sr^{90} and Ca^{45} experiments were comparable in all respects except that they were done at different times, the latter not being started until after the last animal of the former had died. It is highly improbable that the divergent results were due to subtle environmental variations or to genetic change in the mouse colony. A more likely explanation is based on the retention of the radionuclides, the range of the β -rays, and the rate of bone growth.

Strontium-90. The object of the Sr^{90} experiment was to examine the hypothesis that the initial high dose rate immediately after injection is largely responsible for the osteogenic sarcomas that appear many weeks later and that the dose accumulated from the retained radionuclide months later is more or less superfluous as far as tumor induction is concerned. With fractionation it is possible to deliver the same total dose as that given in a single injection and at the same time to limit the maximum body burden to 48% (5 fractions) or 41% (20 fractions). The body burdens of the animals that received a single injection exceeded these levels for 5 and 8 days, respectively, and these mice contained more Sr^{90} than those that were receiving multiple injections during the first 18 days of the experiment. The average difference in the maximum burden during the injection period of the mice receiving 5 and 20 injections was about 10%.

Since the range in bone of the Sr^{90} - Y^{90} β particles is long relative to the size of mouse bone, the fact that the actual deposition sites varied with the three regimens of injection was not very important. This point is illustrated diagrammatically in Figure 19. Since it is not possible to show the radiation in all directions, it is shown in only one in the figure. The injected material is represented as being deposited in the calcifying region proximal to the distal epiphyseal plate of the femur because this bone provides useful reference points for evaluating the range of the radiation.⁽²⁾ At 70 days of age the femur is about 14.6 mm long, the calcifying zone is about 0.3 mm wide, the diameter is less than 1.5 mm, and the cortex is about 0.12 mm thick. The femur is increasing in length at the rate of 0.033 mm/day, and most of the growth occurs at the distal epiphysis. The full-grown femur is about 16.3 mm long.

Figure 19. Proportion of total amount of $\text{Sr}^{90}\text{-Y}^{90}$ in the growing epiphyseal plate of the femur as a function of time after the first injection. Both time and distance are given on the abscissa, the distance being the longitudinal growth of the femur in the specified time. Total shaded area: maximum range of the beta particles (5.5 mm); medium plus heavy shading: average distance traversed by the beta particles (1.3 mm); heavy shading: distance at which the dose rate from a thick plane source in bone drops to $1/2$ (0.69 mm).

The β particles have a maximum range of 1.1 g/cm^2 and the dose they deliver is reduced to half by about two-thirds of a millimeter of bone. In view of these facts and of the dimensions of mouse bone, the exact microscopic location of the Sr^{90} has little effect upon the total volume of bone irradiated or upon dose intensity at any specific site. Therefore, the explanation for the greater carcinogenic efficacy of a single injection as compared to 5 injections, and for 5 as compared to 20, must lie in the temporal relationships of body burden or dose rate. A single injection was most effective because for the first 5 days the dose rate exceeded that of the other injection schedules, and 5 fractions were more effective than 20 because the maximum dose rate during the injection period was about 10%

higher with the former. Comparisons of tumor expectancy when 0.5 and 0.25 $\mu\text{C/g}$ were given in one or in multiple injections indicate that the radiation received during the first week after a single injection accounted for about 36% of the osteogenic sarcomas. Also, the difference of 10% in maximum body burden during the period of injecting 5 or 20 fractions may have been responsible for the observed 16% difference in tumor expectancy.

Calcium-45. The purpose of the duplicate experiment with Ca^{45} was to investigate carcinogenicity with a weak β -emitter that had the same microscopic distribution as Sr^{90} . We had not anticipated that the greater retention of calcium would so greatly decrease the difference in body burden between single and repeated injections. Although the body burden after a single injection exceeded that after repeated injections for 6 days, the maximum difference was only 26% instead of the 52% observed when Sr^{90} had been given. Another point of disparity was that after the fourth day the maximum body burdens with 5 and 20 fractions were very similar.

The range of the Ca^{45} β ray is short compared to the size of mouse bone, as illustrated in Figure 20. As a consequence, the spatial distribution of the dose depends upon the microscopic location of the isotope, and the exact deposition site varied markedly with the three injection schedules. The osteogenic sarcoma data probably can be explained on the basis of the differences in both the spatial distribution and the temporal distribution of the absorbed dose.

Figure 20
Proportion of total amount of Ca^{45} in the growing epiphyseal plate of the femur as a function of time after the first injection. Both time and distance are given on the abscissa, the distance being the longitudinal growth of the femur in the specified time. Total shaded area: maximum range of the beta particles (0.29 mm); medium plus heavy shading: average distance traversed by the beta particles (0.06 mm); heavy shading: distance at which the dose rate from a thick plane source in bone drops to 1/2 (0.036 mm).

Five fractional injections were more carcinogenic than 1 or 20, and 20 fractions were more carcinogenic than 1 injection. A single injection resulted in the intense irradiation of a limited volume of bone. Since the dose rate was reduced to half in 0.036 mm and the total range extended over only 0.290 mm, the dose rate throughout the femur was very uneven. In addition, the femur was growing in length at the rate of about 0.033 mm/day. The physiological factors that caused deposition of Ca^{45} in a particular location would also cause the deposition of stable calcium in that location, with the result that the Ca^{45} deposit was soon buried in solid bone. It is doubtful that osteocytes give rise to osteogenic sarcomas, so that once Ca^{45} is buried it probably is not responsible for many bone tumors. Therefore, the effective carcinogenic life of the nonuniformly deposited Ca^{45} can be measured in days. The experimental data and arguments supporting this view appear in reports by Marshall and Finkel, (2,8) and by Marshall. (9)

Five fractional injections of Ca^{45} produced 5 times as many spots of concentrated radioactivity that were, on the average, $1/5$ as intense as the hotspots from a single injection. Another point of difference is that cells adjacent to the calcified and calcifying bone were potentially exposed to radiation 28 days longer.

Twenty fractional injections could have produced 4 times as many hotspots as 5 injections with an average concentration of Ca^{45} $1/4$ as great if the interval between injections had been long enough. Since there were 5 injections each week during the injection period, and since growth at a specific site proceeds for more than 1 day, there was chance for the Ca^{45} administered on successive days to be deposited in the same place. As a consequence, the actual number of hotspots was less than the possible maximum, and their average intensity was greater than the possible minimum. In spite of these considerations, however, with 20 injections a greater volume of tissue was irradiated than with 5 injections, and the dose rate was more uniform throughout the bone. Another point is that the actual exposure time with 20 fractions was 3 days shorter than the exposure time with 5.

Taking these various factors into account, we suggest that dividing the total amount of Ca^{45} into 5 fractions produced more osteogenic sarcomas than a single injection because the volume of tissue irradiated was 5 times greater and the effective exposure time was 28 days longer. These factors more than compensated for the fact that each hotspot contained, on the average, only $1/5$ as much Ca^{45} . In view of the repeated observation that the carcinogenic response to bone-seekers increases approximately as the square of the amount injected, (8,10) reducing the dose to $1/5$ could be expected to reduce the number of tumors to $1/25$.

Twenty fractions were less efficient than 5, a result that can be explained on the basis of an appropriate balance between the increase in the

volume of tissue irradiated and a decrease in both the exposure time and in the intensity of the hotspots. The fact that 20 exposures were more efficient than 1 implies that the greater volume of tissue irradiated and the prolonged period of exposure still more than compensated for the reduced intensity of the hotspots.

Phosphorus-32. The P^{32} experiment of Blackett⁽⁶⁾ and Lamerton⁽⁷⁾ introduced another variable that we have not yet tested adequately in mice, namely the half-life of the radionuclide. Since the P^{32} β ray has a maximum range in bone of 4 mm, the spatial distribution of the dose in mouse bone would not be very different from that described for Sr^{90} - Y^{90} . Rats were used in this study, so the dose was not as uniform throughout the bone as it would have been in the smaller mouse. A pertinent observation relating to this point is that of Vaughan and Owen:⁽¹⁾ in the rabbit, which again is much larger than the rat, the ratio of maximum to minimum dose rate in bone from injected Sr^{90} was about 40. An uneven distribution of the dose from P^{32} in the rat would favor an increase in carcinogenicity with fractionation because a larger volume of tissue is irradiated with multiple injections.

Still more important, however, is the fact that excretion reduces the amount of P^{32} in the rat to about 51% 14 days after administration, at which time about 62% of the retained P^{32} is in the skeleton.⁽¹¹⁾ Physical decay in 14 days reduces the amount further, so that only 25.5% of the injected P^{32} remains. We have estimated that the body burden of Blackett's and Lamerton's rats that received a single injection exceeded that of the other animals until the second injection (day 14) and that after the third injection (day 28) the latter animals contained much more P^{32} than those that had received a single injection. The maximum difference occurred at the time of the last injection (day 54), when the body burden after a single injection was 2% and the rats receiving multiple injections contained 25%. Consequently, on the basis of body burden, or temporal distribution of the dose, it would be anticipated that more tumors would arise with fractional injections, and this indeed was the case.

2. Continuous Exposure

Mice were maintained on a constant diet of Sr^{90} , with the result that the amount of Sr^{90} they contained increased gradually, and the distribution of the absorbed dose both in space and in time was as uniform as could be attained. The radionuclide was incorporated in the food in amounts ranging from 15 to 0.01 $\mu\text{c/g}$ Ca according to the method described earlier.⁽¹²⁾

The experiment was started with adult CF No. 1 females that were permitted to mate two days after they had been placed on the Sr^{90} food. They and their offspring were maintained on the same food as long as they lived. The male offspring, except those on the 15 $\mu\text{c/g}$ calcium food, were used for radioanalysis and autoradiography.

Results

The current osteogenic sarcoma data are summarized in Table 3. One animal is still alive, and the histologic examination of all the material has not been completed.

TABLE 3

Sr^{90} ingestion: current osteogenic sarcoma data

Initial age	Sex	$\mu\text{c Sr}^{90}/\text{g Ca}$	Number of mice	Tumor incidence*	Tumor expectancy**
~150 days	F	15	44	1.07	1.3
		10	41	0	0
		5	21	0	0
		2.5	46	0.043	0.03
		1.0	20	0	0
		0.1	39	0.026	0.02
		0.01	31	0.032	0.01
		0	46	0	0
		15	50	1.26	1.9
		10	95	0.28	0.71
<u>in utero</u>	F	5	53	0.06	0.10
		2.5	129	0.008	0.014
		1.0	49	0	0
		0.1	93	0	0
		0.01	71	0	0
		0	146	0	0
		15	57	0.35	0.52
		0	45	0	0
	M	15	57	0.35	0.52
		0	45	0	0

*Tumors/mouse.

**Number of tumors to come divided by number animals alive calculated at 10-day intervals and averaged from 150 days after the start of the experiment until less than 3 mice remained alive.

Only the food containing the greatest concentration of Sr^{90} ($15 \mu\text{c/g}$ calcium) unquestionably increased the incidence of osteogenic sarcomas among the group first exposed to the diet at about 150 days of age. The number of tumors obtained with the food containing $2.5, 0.1$, and $0.01 \mu\text{c Sr}^{90}/\text{g}$ calcium was within the control range. The female mice first exposed to the $15, 10$, or $5 \mu\text{c}$ food before birth had more tumors than normal, but the results with the $2.5 \mu\text{c}$ food were within the control limits. The male mice that ate the $15 \mu\text{c}$ food had only $1/4$ as many malignant bone tumors as their female littermates.

The mortality rates with osteogenic sarcoma of the female mice exposed to Sr^{90} from the time of conception are presented in Figure 21. The daily rate was derived from rates over a 50-day interval calculated every 10 days as long as 3 or more mice remained alive. The first tumor death occurred at 325 days when the dietary level was $15 \mu\text{c/g Ca}$, at 457 days when it was $10 \mu\text{c/g Ca}$, and at 717 days when it was $5 \mu\text{c/g Ca}$.

Figure 21. Daily rate of death with osteogenic sarcoma as a function of age among female mice maintained from the time of conception on food containing 15, 10, or $5 \mu\text{c Sr}^{90}/\text{g Ca}$

The difference in response between the male and female littermates maintained on the highest-level food is illustrated in the mortality rate with osteogenic sarcoma (Figure 22). The first tumor death among the males occurred 84 days later than in the females, but the rate among the females at that time was still low.

Figure 22

Daily rate of death with osteogenic sarcoma as a function of age among male and female mice maintained from the time of conception on food containing $15 \mu\text{c Sr}^{90}/\text{g Ca}$

The difference in tumor response related to the age at which the Sr^{90} was first ingested is illustrated in Figure 23. The earliest tumor deaths occurred at about the same time among the dams and their female offspring, but the slopes of their disease-specific mortality rates were quite different. The tumor data from the standard long-term toxicity experiment with Sr^{90} that are most similar to the consequences of eating food containing $15 \mu\text{c Sr}^{90}/\text{g Ca}$ are those resulting from the injection of $0.44 \mu\text{c/g}$. (10,13) Those data are included in Figure 23, and the body

burdens of these three groups of mice are compared in Figure 24. The animals that had been exposed to Sr^{90} since conception contained $2.3 \mu\text{c}$ when they were 100 days old, and further increase was negligible. Their dams contained about $0.75 \mu\text{c}$ after they had been eating Sr^{90} for 100 days, and their body burdens continued to increase gradually. In contrast, the animals that had received an injection of $10.2 \mu\text{c}$ ($0.44 \mu\text{c/g}$) when they were 70 days old contained $2.3 \mu\text{c}$ 58 days later, and after 75 days they contained less Sr^{90} than the mice on the continuous Sr^{90} diet. However, their body burdens exceeded that of the older mice for 450 days.

Figure 23

Daily rate of death of female mice with osteogenic sarcoma as a function of time after first exposure to Sr^{90} . One group received a single intravenous injection at 70 days of age, and the other 2 groups were maintained on food containing $15 \mu\text{c Sr}^{90}/\text{g Ca}$, 1 from conception and the other from about 150 days of age.

Figure 24

Sr^{90} body burden as a function of time: 1) after the injection of $0.44 \mu\text{c/g}$ ($10.2 \mu\text{c}$ total) at 70 days of age, 2) after starting to eat Sr^{90} at about 150 days of age, and 3) after birth when maintained on a continuous Sr^{90} diet. The vertical lines denote the limits of one standard error.

Discussion

Tumor expectancy of the female mice exposed to Sr^{90} since conception decreased as the concentration of Sr^{90} in the diet decreased. As is the case after injection, the carcinogenic response seemed to be related to the square, or perhaps to a somewhat higher power, of the dose. A decrease in dietary concentration from 15 to $10 \mu\text{c/g Ca}$ ($1/1.5$) decreased tumor

expectancy to $1/2.7$, and a decrease from 10 to $5 \mu\text{c/g Ca}$ ($1/2$) decreased tumor expectancy to $1/7$. In the first case the carcinogenic responses differ by the 2.5 power of the dose, and in the second by the 2.8 power of the dose.

The disease-specific mortality rates show a difference in the time of appearance of the osteogenic sarcomas associated with dietary level (Figure 21). If we assume that food containing $5 \mu\text{c Sr}^{90}/\text{g Ca}$ is close to the minimum diet for obtaining measurable tumor response in the mouse, then the minimum carcinogenic body burden is about $0.8 \mu\text{c}$. This level would be reached at 100 days on the $5 \mu\text{c}$ diet, at 50 days on the $10 \mu\text{c}$ diet, and at 25 days on the $15 \mu\text{c}$ diet. Since these time differences are much smaller than the apparent 200-day difference in tumor time when the dietary level is 15 or $10 \mu\text{c}$, dose rate alone is not the whole answer, and the contribution of at least part of the integral dose to tumor induction needs to be considered when suitable data are available.

Comparison of the tumor response of male and female mice maintained continuously on food containing $15 \mu\text{c/g Ca}$ shows a striking difference between the sexes (Figure 22). This was the first experiment in which we kept CF No. 1 males for long-term study, and it points out the importance of not ignoring the possibility of sex differences in carcinogenesis. With individual caging we will be able to do further work with the pugnacious CF No. 1 male mouse.

The difference in tumor response between the dams and their female offspring can be explained, at least in part, on the basis of their body burdens. Adult mice fed $10 \mu\text{c Sr}^{90}/\text{g Ca}$ would not reach the $0.8 \mu\text{c}$ level assumed above to be the minimum to give a measurable response for 420 days. It is not surprising, therefore, that there were no tumors among the adult mice on that diet because only 15 were alive at 420 days, and 12 of these died before 570 days, a time interval which would provide for the usual 150-day latent period between "induction" and death with osteogenic sarcoma. However, if this argument is valid and the dams can be compared in this fashion to their female offspring, then there were too many tumors among those dams that were fed $15 \mu\text{c Sr}^{90}/\text{g Ca}$ (Table 3 and Figure 24). These confusing relationships require further study, which will be done when the data are complete.

Equivalent carcinogenic amounts of injected and ingested Sr^{90} were selected for comparison so that the relative contribution of the initial high dose and the retained dose could be evaluated. The body burdens in Figure 24 should provide the clue, but there are still too many unknowns. For that reason we have turned to experimental techniques that permit a greater manipulation and control of the delivered dose.

3. Time-limited Irradiation

The bone-seeking radioisotopes are excellent carcinogens, but they present many obstacles to discovering why and how they induce osteogenic sarcomas. These obstacles derive from the fact that the dose delivered by radionuclides varies both in space and in time, and most of them continue to irradiate the animal long after the neoplastic change has taken place. As a result, it has been extremely difficult to arrive at any reliable and universally acceptable estimate of the absorbed dose required for carcinogenesis. We have two experiments in progress that should simplify some of the problems related to residual body burden.

Yttrium-90

Y^{90} possesses very useful qualities for studying radiocarcinogenesis. It is deposited primarily in bone, (14) it emits an energetic β ray, which, in fact, is largely responsible for the toxicity of Sr^{90} , and it has a half-life of only 64 hr. We hoped that by maintaining various levels of Y^{90} in the body for varying lengths of time we would be able to separate and, thus, recognize the contributions of the dose rate and the accumulated dose.

Figure 25. Y^{90} body burden as a function of time after the first injection. Thirty per cent of the initial amount was injected 11, 30, 52, 76, and 100 hours after the first injection.

The first step was to determine a carcinogenic amount of Y^{90} . In some earlier experience with this isotope 10-12 $\mu\text{c/g}$ given in a single injection produced a few osteogenic sarcomas, but this amount also killed 50% of the mice within 30 days. (15, 16) We assumed, accordingly, that fractionation would be more carcinogenic than a single injection. Seventy-day-old CF No. 1 female mice were given repeated intravenous injections of Y^{90} according to a schedule that would maintain their body burdens for 5 days between 70% and 100% of 1.0, 0.5, and 0.25 $\mu\text{c/g}$, as shown in Figure 25. Radioactive decay quickly eliminated the Y^{90} after the last injection.

The experimental routine includes weekly roentgenographic examination of the bones of the living mice. This procedure makes it possible to separate the rate of appearance

of osteogenic sarcomas from mortality rate. The data in Table 4 include tumors in dead animals as well as tumors in animals alive at the moment. So far, the $0.5 \mu\text{c/g}$ maximum level is as carcinogenic as $1.0 \mu\text{c/g}$, and the lowest injected dose level also has induced a few osteogenic sarcomas.

TABLE 4

Y^{90} injection: results at 590 days

Maximum body burden, $\mu\text{c/g}$	Total amount injected, μc	Number of mice	Mortality, %	Osteogenic sarcomas	
				Incidence, %	Tumors/ mouse
1.0	60	30	53.3	13.3	0.13
0.5	30	60	48.3	13.3	0.15
0.25	15	120	45.0	4.2	0.05
0	0	120	52.5	0.8	0.008

There is a striking difference between the early roentgenographic appearance and growth of these tumors and the tumors induced by Sr^{90} .⁽¹⁷⁾ The average length of time between the first roentgenographic recognition of an osteogenic sarcoma and death of the animal was 38.5 days when Sr^{90} was used. The current average time for the Y^{90} osteogenic sarcomas is 170 days. The lesions seem to remain stationary for weeks, waiting for an additional stimulus before beginning progressive growth. This curious result introduces the possibility that a two-stage hypothesis of tumor production might apply to radiation-induced osteogenic sarcomas.

External Exposure

Exposure to X-rays is even more amenable to manipulation than exposure to Y^{90} . In our first attempt to induce osteogenic sarcomas with X-rays, we exposed one hind leg of 70-day-old CF No. 1 female mice to 2000-5000 r.⁽¹⁸⁾ Tumors appeared in all groups, but radiation damage was so severe that the carcinogenic response was impaired. In the next experiment smaller doses were given, and both hind legs were exposed to 250 KVP X-rays at a dose rate of 58-59 r/min.

The current results are given in Table 5. Seven osteogenic sarcomas have appeared among the 750 mice that received 250 r. Of these tumors, 5 were in the irradiated area and 2 were not. Precise calculations have not been made, but a reasonable estimate is that the 250 r delivered about 500 rads to the bone.

It is particularly interesting that the present data show an increase in tumors almost equal to the increase in dose. In fact, the data from this partially completed study indicate that the carcinogenic response (tumors per mouse) varies as the 1.13 power of the dose, a value that is almost

linear. If this relationship continues to hold when all the data have been collected, it will provide a basis for examining the reason for the frequent observation that the incidence of osteogenic sarcomas increases as the square, or even the cube, of the injected amount of a bone-seeking radioisotope. The explanation should rest upon the fact that no isotope can deliver an absolutely uniform dose throughout the entire bone, and bone has three dimensions. When an X-ray exposure dose is doubled, the absorbed dose is uniformly doubled. When the amount of radioisotope is doubled, the absorbed dose is doubled only in that volume of tissue that lies within the range of the emitted particles. The critical information still wanting concerns the definition and location of the region that must be irradiated if neoplastic change is to occur.

TABLE 5

External exposure: results at 442-575 days

Exposure dose, r	Number of mice	Mortality, %	Osteogenic sarcomas		
			In irradiated area		Outside of irradiated area, %
			Incidence, %	Tumors/mouse	
1000	300	63.7	3.0	0.033	0.3
625	750	68.0	1.6	0.019	0.13
250	750	68.8	0.7	0.007	0.27

Summary

1. A single injection of Sr^{90} produced more osteogenic sarcomas in female mice than the same total amount given in 5 fractions, and 5 fractions were more carcinogenic than 20. These results are explained on the basis of the temporal distribution of the absorbed dose. Approximately 36% of the osteogenic sarcomas produced by a single intravenous injection of Sr^{90} may have been due to the high dose rate during the first week after injection.
2. A single injection of Ca^{45} produced fewer osteogenic sarcomas than the same total amount given in 5 or 20 fractions, and 5 fractions were more carcinogenic than 20. These results are explained on the basis of both the temporal and spatial distribution of the absorbed dose.
3. Continuous exposure to dietary Sr^{90} from the time of conception resulted in the production of osteogenic sarcomas when the food contained 5 or more $\mu\text{c Sr}^{90}/\text{g Ca}$. The body burden of the mice on the 5 μc food reached 0.8 μc when they were 100 days old, and there was very little increase thereafter. Death with tumors occurred later when the level of dietary Sr^{90} was lower.

4. Male mice on the 15 μc food had only 1/4 as many osteogenic sarcomas as their female littermates.
5. Mice placed on dietary Sr^{90} when they were about 150 days old had an increased incidence of osteogenic sarcomas when the food contained 15 μc Sr^{90} /g calcium. Their Sr^{90} content was about 0.75 μc 100 days after they started on the diet, and it continued to increase slowly.
6. An Y^{90} body burden was maintained between 70% and 100% of 1.0, 0.5, or 0.25 μc /g for 5 days by a series of repeated injections. Malignant bone tumors have appeared at each level. The average time between roentgenographic recognition of the Y^{90} -induced tumors and death of the host is much longer than is the case with Sr^{90} -induced tumors.
7. Exposure of the hind legs of mice to 1000, 625, and 250 r of X-rays has produced a few osteogenic sarcomas. The preliminary results suggest a linear relationship between dose and response in this dosage range in contrast to the results with injected bone-seeking radionuclides.

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TOXICITY AND METABOLISM OF RADIONUCLIDES.
V. PROGRESS REPORT FOR PERIOD ENDING JUNE 29, 1963

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Pathologic Alterations in Beagle Dogs Injected Intravenously
with Cs¹³⁷ at the LD_{50/30} Level

In this section are described the lesions observed in 24 beagles of 4 age groups (5 and 13 months, 4 and 5 years) that died after intravenous injection of Cs¹³⁷ (2000 to 4000 $\mu\text{c/kg}$), as well as those observed in 2 beagles sacrificed 1 and 2 weeks after intravenous injection of Cs¹³⁷ (3140 and 3180 $\mu\text{c/kg}$).

The procedures used at necropsy and for the preparation of tissues for histologic examination were similar to those described previously.⁽¹⁾ Table 6 lists the number of animals in each age group, the amount of Cs¹³⁷ injected and the days to death.

Gross Pathology

The pathologic findings at necropsy are summarized in Table 7. Hemorrhages were found in all organs and tissues with few exceptions; their number and extent varied considerably. Since the small hemorrhages (petechial and ecchymotic) occurred in all or nearly all organs and tissues, they are omitted from Table 7 for the sake of brevity.

Hemorrhagic extravasations (lesions measuring 125 mm and above) and effusions, were limited to the dermis and subcutis of the head and neck, the mucosa of the intestine, and the body cavities. Those involving the dermis and subcutis of the head occurred almost exclusively in the 4- and 5-year-old dogs (Figure 26), and were associated with necrotic lesions at the roots of the teeth. Hemorrhagic extravasations associated with venipuncture were observed in the subcutis of the cervical region in all except two dogs (0398 and 0419).

In all except the 5-year-old dogs, hemorrhagic extravasations were seen on the mucosal and serosal surfaces of the rectum and colon. Blood loss from the intestine was considerable as indicated by the quantity of blood within the lumen of the large intestine. Similar but generally less severe lesions occurred in the small intestine in some animals. In most of the 5-year-old dogs hemorrhages were concentrated in the wall of the duodenum. Gastric hemorrhages were severe in the higher age groups (Figure 27).

TABLE 6
Survival of beagles injected with Cs137

Dog number	Sex	Age	Dose injected, $\mu\text{C/kg}$	Days to death
Experiment 002				
0107	M	Adult	1990	64
0121	M	"	2040	27
0124	M	"	1580	
			1040	
0114	M	Adult	930	
			1010	
			1130	
0128	M	Adult	1082	
			990	
			1010	
Experiment 004				
0397	M	5 months	3670	26
0398	M	"	3790	
0399	M	"	3240	27
0401	F	"	3680	
0404	F	"	3650	
0470	M	"	3850	
0471	M	"	3760	
0472	F	"	3650	
0473	F	"	3790	
0474	F	"	3600	
0485	M	"	3640	
0486	M	"	3590	
0487	M	"	3700	39
0491	F	"	3650	
0492	F	"	3760	
0052	M	13 months	2108	2
0053	M	"	2110	0
0054	M	"	1982	
0055	M	"	2107	
0081	M	"	1854	
0246	F	"	2430	
0247	F	"	2160	
0256	F	"	2270	
0257	F	"	2230	
0258	F	"	2320	
0237	F	"	3590	28
0241	F	"	3690	33
0243	M	"	3330	35
0239	M	"	3360	
0366	F	"	3170	
0367	M	"	3090	36
0369	M	"	3100	28
0390	F	"	3310	
0391	F	"	3340	
0392	F	"	3450	
0393	F	"	3410	
0416	F	"	4246	24
0417	M	13 months	4309	27
0418	M	"	4281	26
0419	M	"	4140	27
0435	M	"	3399	
0436	M	"	3388	
0437	M	"	3468	
0438	M	"	3532	
0428	F	"	2731	
0430	F	"	2650	
0439	M	"	1704	
0441	F	"	2704	52
0443	M	"	1729	
0444	M	"	1739	
0449	M	"	2650	
0450	M	"	2844	
0451	M	"	2700	
0452	M	"	2684	
0453	F	"	1665	
0454	M	"	1651	

TABLE 6 (Contd.)

Dog number	Sex	Age	Dose injected, µc/kg	Days to death
Experiment 004 (Contd.)				
0313	F	18 months	3180	
0316	F	"	3140	
0046	F	48 months	3740	22
0048	F	"	3640	21
0069	M	"	3710	25
0071	M	"	3700	23
0072	M	"	3700	23
0060	F	66 months	3390	24
0063	F	"	3320	26
0064	M	"	3290	29
0065	M	"	3400	28
0080	M	"	3270	24
0039	F	Adult	250	25
0103	F	"	250	24
0100	F	"	250	28

TABLE 7

Summary of gross pathologic findings in beagle dogs of 4 age groups*

Dog number	Thorax and contents	Lung	Heart	Pericardium and contents	Peritoneum and contents	Liver	Stomach	Small intestine	Large intestine	Skeletal muscle	Skin (dermis and subcutis)
5 month age group											
0398	-	E	-	-	-	C, Cy, N	-	-	He ₂ , U	Hi ₂	He ₁ , Hi ₁
0401	Hi ₃	E, Hi ₃	Hi ₃	Hi ₃	-	C	-	Hi ₂	He ₂ , U	Hi ₂	He ₁
0487	-	E	-	-	-	C, Cy, N	-	Hi ₂	He ₂ , U	Hi ₂	He ₁ , Hi ₁
13 month age group											
0107	HO, Hi ₂	E, Hi ₃	Hi ₃ , Hi ₁	HO, Hi ₂	HO	C	-	He ₂ , U	-	Hi ₂	He ₁ , Hi ₁
0121	HO, Hi ₂	E, Hi ₂	Hi ₃ , Hi ₂	HO, Hi ₂	HO	C, Cy, N	-	He ₂ , U	He ₂ , U	He ₂ , Hi ₃	He ₁
0237	HO, Hi ₂	E, Hi ₃	Hi ₃ , Hi ₂	HO, Hi ₂	HO	C	-	-	He ₂ , U	He ₁ , Hi ₃	He ₁
0241	HO	E, Hi ₃	Hi ₃ , Hi ₂	HO, Hi ₂	HO	C	-	-	He ₂ , U, R	Hi ₂	He ₁
0243	HO, Hi ₂	E, Hi ₂	Hi ₂ , Hi ₁	HO, Hi ₂	HO	C, R	-	-	He ₂ , U	Hi ₃ , Hi ₂	He ₁
0367	HO	E, Hi ₂	Hi ₃	Hi ₃	-	C	-	-	He ₂ , U	Hi ₃ , Hi ₂	He ₂ , Hi ₂
0369	HO	E, Hi ₂	Hi ₃ , Hi ₁	HO, Hi ₂	HO	C, Cy, N	-	-	He ₂ , U	Hi ₃ , Hi ₂	He ₂ , Hi ₂
0416	HO	E, Hi ₂	Hi ₃	HO	-	C, Cy, N	-	-	He ₂ , U	Hi ₂	He ₁
0417	HO	E, Hi ₂	Hi ₂	-	-	C	-	-	He ₂ , U	Hi ₂	He ₁
0418	HO	E, Hi ₂	Hi ₂	HO	HO	-	Hi ₂	-	He ₂ , U	Hi ₂	He ₁ , Hi ₂
0419	HO	E, Hi ₂ , N	Hi ₂ , Hi ₂	He ₁ , HO	-	C	Hi ₁	-	He ₂ , U	Hi ₂	-
4 year age group											
0046	-	E	-	-	HO	C, N	-	-	He ₂ , U	He ₂ , Hi ₂	He ₁
0048	-	E	-	-	-	C	Hi ₂ , U	Hi ₃ , Hi ₃	Hi ₂ , U	Hi ₂	He ₁
0069	-	E, He ₁	Hi ₂	-	-	C	-	-	He ₂ , U	Hi ₂	He ₁
0071	-	E, Hi ₂	-	-	-	C	Hi ₂ , U	Hi ₂	He ₂ , U	Hi ₂	He ₁
0072	-	E	Hi ₂	-	-	C	-	-	He ₂ , U	He ₁ , Hi ₂	He ₁
5 year age group											
0060	-	E, Hi ₂	Hi ₂	-	-	C	Hi ₃ , U	He ₂ , U	-	Hi ₂	He ₂ , N
0063	-	E, Hi ₂	-	-	-	C	-	He ₂ , U	Hi ₂ , U	He ₂ , Hi ₂	He ₂ , N
0064	-	E, Hi ₂	-	-	-	C	-	He ₂ , U	-	He ₁	He ₂ , N
0065	-	E	-	-	-	C	-	He ₃ , U	-	He ₁ , Hi ₂	He ₂ , N
0080	-	E	Hi ₁	-	-	C	He ₁ , U	He ₂ , U	-	Hi ₂	He ₂ , N

*The recording of hemorrhages under 5 mm was omitted because of their general distribution and for the sake of brevity.

C - congestion

Cy - cysts

E - edema

N - necrosis

R - rupture

U - ulcers

He - hemorrhages of sizes over 125 mm

HO - hemorrhage into a body cavity or space

HI - hemorrhages of sizes between 5 and 25 mm

Hi - hemorrhages of sizes between 25 and 125 mm

He - hemorrhages of sizes over 125 mm

HO - hemorrhage into a body cavity or space

1 - one area of hemorrhage

2 - sparse hemorrhages

3 - generalized hemorrhage

Figure 26. Head of a 5-year-old dog (0080). Hemorrhagic extravasations involve the dermis and subcutis of the head and neck. E, eyes; G, gum.

Figure 27. Thoracic and abdominal organs of a 13-month-old dog (0369). Hemorrhages occur in the heart and lungs. There are cysts on the liver and hemorrhages in the intestines. H, heart; L, lung; l, liver.

Hemothorax, hemopericardium, hemoperitoneum, and hemorrhagic effusions into the intercostal muscles, mediastinum, diaphragm, pericardium, heart and lungs were seen commonly among the 13-month-old animals (Figure 28), and occurred in animals of other age groups as well (Table 7). Two of the 5-month-old dogs (0398 and 0487) were free of thoracic lesions. Large hemorrhages (25 to 50 mm) also occurred throughout the skeletal muscle; in a few animals (0063, 0065, 0072 and 0121) they approximated 100 to 120 mm in greatest dimension.

Figure 28. Abdominal organs of a 4-year-old dog (0048). Hemorrhages of varying size appear in the stomach and intestines. S, stomach; L, lung; 1, liver.

The testes were the sites of large hemorrhages in most of the males. Large hemorrhages were seen occasionally in other organs; for example in the kidneys in one dog (0080) and in the cerebral ventricles in another (0072).

The following changes were consistently found in animals of all age groups: spleens, small, contracted and homogeneously dark reddish-purple; lymph nodes, dark reddish-purple, edematous and enlarged; tonsils,

ulcerated, edematous and necrotic; bone marrow, dark reddish-purple and fatty; liver, congested; intestines, mucosal ulceration; lungs, edematous and hyperemic.

Multiple necrotic foci with or without cyst formation were found in the livers of several dogs (0046, 0369, 0416 and 0487). Ulcers were seen in the mucosa of the nasal sinuses in two (0107 and 0398).

Histopathology

Since small foci of hemorrhage were observed microscopically in all or nearly all organs, they will be mentioned only under the cardiovascular system. Unless otherwise stated, the alterations described below are those seen in the majority of animals.

Respiratory System

The mucosae of the nasal sinuses were ulcerated and necrotic. In the lungs the alveoli contained proteinaceous material, erythrocytes, fibrin, hemosiderocytes, plasma cells, macrophages with phagocytized lipid droplets and free bacteria. Macrophages with phagocytized lipids and blood pigment were seen within the alveolar and bronchial walls of the interstitial tissues. The alveolar capillaries were distended with erythrocytes.

Cardiovascular System

Myopathy, vascular degenerative change, thrombi and infarcts were observed in the myocardium. Areas of atrophy, degeneration and necrosis occurred in varying degrees in all animals. Evidence of altered glycogen metabolism was observed in myocardial muscle. Abundant glycogen was present between the sarcolemma and the fiber.

Thrombi occurred within the venules of the myocardium as well as of all or nearly all organs in association with deposition of mucopolysaccharides within the vessel wall. The thrombi consisted of fibrin, granular and hyaline masses and round to oval vacuolated bodies; the latter stained with periodic acid Schiff, phosphotungstic acid hematoxylin and Sudan black B, and were undigested by diastase. The apparent encapsulation of the round bodies was considered to be a surface tension phenomenon due to the combination of lipids, protein and mucopolysaccharides (Figures 29 and 30). Such bodies measured from 10 to 100 μ . Gram-negative and Gram-positive bacteria were found intravascularly in the tissues of several animals (0046, 0121, 0237, 0241, 0369 and 0487) (Figure 31).

Figure 29. Liver of dog 0048. The vein in the center field contains a spherical acidophilic structure with an internal granular pattern (hematoxylin and eosin).

Figure 30. Same field as in Figure 29. The structure contains numerous vacuoles (hematoxylin and eosin).

Figure 31. Bone marrow of dog 0046. Gram-positive and -negative bacilli are both within the cytoplasm of fixed macrophages and free within sinusoids (Brown and Brenn).

Digestive System

Liver. The sinusoids and the central veins of the liver were markedly distended with erythrocytes. The intima of the central vein was edematous and the endothelium was either absent or disrupted. Hepatic cells near the central vein were either pyknotic or absent. Fat droplets were present in all hepatic cells but were most abundant near the central vein. Throughout the lobules hepatic cells were seen with vacuolated nuclei and acidophilic intranuclear crystalloids (Figures 32 and 33). Bile pigment and ceroid occurred in the cytoplasm of hepatic cells. Kupffer cells contained erythrocytes, hemosiderin, lipid and mucopolysaccharides. In the animals with grossly visible necrotic lesions, extensive areas of nonexudative necrosis were associated with aggregates of bacteria; cysts were formed within the necrotic areas.

Figure 32. Liver of dog 0072. In center field there is an intranuclear crystalloid which stretches the nuclear membrane of the hepatic cell (hematoxylin and eosin).

Figure 33. Same section as in Figure 32. In center field is a vacuolated nucleus of a hepatic cell (hematoxylin and eosin).

Gastrointestinal Tract. The mucosae of tongue, esophagus, stomach and intestine were ulcerated and hemorrhagic. Mitotic figures were sparse at the base of the intestinal crypts. The epithelium over the villi was disrupted or lacking and the lamina propria was the site of hemorrhage, necrotic foci and bacterial aggregates. The lymphoid tissue of Peyer's patches was replaced by erythrocytes, plasma cells and hemosiderocytes.

Hematopoietic System

Bone Marrow. The marrow was aplastic in most of the dogs or was hypoplastic as in the 5-month-old dogs. Only abnormal megakaryocytes were seen. Mitotic figures were infrequent. The marrow cavity contained fat cells, erythrocytes, fibrin, plasma cells, hemosiderocytes, bacteria and macrophages containing lipid (Figure 34). In the young animals foci of myelopoiesis were noted along the endosteum.

Spleen. Lymphoid tissue was depleted or sparse. Reticulum cells, plasma cells and hemosiderocytes, with many pyknotic cells interspersed, surrounded the central arteriole. In some animals there was a narrow rim of lymphocytes. Large accumulations of hemosiderin appeared throughout the spleen. The sinuses contained erythrocytes, acidophilic granular material, hemosiderocytes, plasma cells and an occasional megakaryocyte.

Lymph Nodes. Lymphoid tissue was hypoplastic; the sinuses were markedly distended with erythrocytes. Megakaryocytes were seen occasionally within the sinuses.

Tonsils. These contained extensive areas of necrosis, multiple deep ulcers, and bacteria.

Thymus. The thymus gland was identified only in the 5- and 13-month-old dogs. The tissue was markedly edematous; the thymocytes were markedly reduced in number, being replaced in part by plasma cells.

Endocrine System

Pituitary. There was a relative increase in the number of acidophils in the pars distalis. The sinusoids were distended with proteinaceous material. Nonciliated cysts containing similar proteinaceous material were present in the pituitary glands of the 4- and 5-year-old dogs.

Thyroid Gland. The colloid-containing acini in the thyroid gland were markedly reduced in number. Multiple cysts were noted in the glands of the 4-year-old and older dogs.

Figure 34. Bone marrow of femoral metaphysis (dog 0046). Plasma cells, mast cells, fat cells and erythrocytes appear in center field (Giemsa).

Adrenal Gland. Foci of necrosis occurred in the cortex and in the medulla. Lipid droplets were patchy in distribution within the zona fasciculata.

Urinary System

Kidney. Animals of all age groups exhibited glomerular and tubular lesions of varying degrees. The tubular lesions were considered primary and consisted of areas of acellularity; the lumen contained casts of sloughed epithelial cells and erythrocytes. Fatty change involved all parts of the nephron, including the glomerular tuft (Figure 35). Glomerular lesions consisted of a diffuse reduction in the number of nuclei in the tuft and distension with erythrocytes.

Urinary Bladder and Urethra. Both in the urinary bladder and in the urethra the lamina propria was ulcerated and hemorrhagic, with bacterial invasion.

Reproductive System

Testis. The testes of several of the adult animals (0069, 0071, 0107, 0243 and 0367) were atrophic; pathobiotic Sertoli and type A spermatogonial cells were the only cells remaining (Figure 36). In the other males, varying degrees of cell damage were noted. Mitotic figures were sparse, abnormal cells were numerous and in many tubules the lumen contained necrotic debris and proteinaceous material.

Central Nervous System

Brain. Edema and focal hemorrhages were generalized. In one dog (0072), in which the lateral ventricles were distended with clotted blood, the cerebral tissue was compressed.

Musculoskeletal System

The skeletal muscles contained areas of atrophy, degeneration and necrosis. Degeneration was both hyaline and granular. Evidence of altered glycogen metabolism was observed in skeletal muscle to a more marked degree than in myocardial muscle. Abundant glycogen was present in the form of a cuff between the sarcolemma and the fiber.

Sacrificed Dogs

Histologic observations in the tissues of the 2 dogs sacrificed at 1 week (0313) and at 2 weeks (0316) after intravenous injection with Cs¹³⁷ were limited to the cervical lymph nodes, spleen, liver and kidney.

Figure 35. Kidney of dog 0487. The glomerular tufts contain numerous lipid droplets (oil Red O)

Figure 36. Testis of dog 0107. Pathobiotic Sertoli cells and type A spermatogonial cells line the tubules (hematoxylin and eosin).

Changes of questionable significance included cytoplasmic vacuolation and a relative increase in plasma cells, in hemosiderocytes and in erythrophagocytosis in the spleen and lymph nodes, as compared with the control (0314) (Figures 37 and 38).

Figure 37. Spleen of dog 0316 which was sacrificed 2 weeks after injection with Cs-137. Vacuolation is prominent in the pulp of the spleen (hematoxylin and eosin).

Figure 38. The same section as in Figure 37. Aggregates of hemosiderocytes occur in discrete foci (hematoxylin and eosin).

Deaths in Control Dogs

During the past year there were 56 deaths; the causes are listed by age groups in Table 8. Five deaths occurred among the dogs on the semisynthetic diet, the rest occurred among dogs on the standard diet.

TABLE 8

Causes of death in nonradioactive dogs
on standard and semisynthetic diets

Age of dog	Cause of death	Number of dogs
Adult	Lymphosarcoma of intestine	1
	Toxemia of pregnancy	1
	Metastatic calcification	1
	Uremic arteritis	1
Young*	Hepatitis	1
	Hemorrhagic enteritis, coccidiosis	1
	Trauma	3
Newborn*	Purpura	7
	Pneumonitis	11
	Inhalation pneumonia	1
	Peritonitis, umbilical infection	8
Stillborn*		20

*One animal each in the young and newborn and 3 in the stillborn groups were on the semisynthetic diet. The remainder were on the standard diet.

Semisynthetic Dog Diet**

Twenty-one dogs are being maintained upon the semisynthetic diet previously described (CASD). During the past 6 months, 6 females came into heat. Of these, two were bred (0004 and 0006). Breeding attempts with 3 (0284, 0272, 0273) were unsuccessful, and the sixth (0045) was not bred because she had produced a litter of puppies within the last 6 months. In May, 1963, 1 of the 2 females (0006) bred whelped a litter of three pups; it was her second litter on the semisynthetic diet. Two of the 3 were stillborn and showed areas of massive hemorrhage in skeletal muscle and in the body cavities; this condition has also occurred in the stock dogs and is

**This work was performed by Mrs. Lillian V. Kaspar.

more thoroughly discussed in the pathology section; the third (0641) survives to date and is apparently normal. The dam required a cesarean section the last time she whelped; this time, however, delivery was normal. The second dog (0004) should whelp her litter in August.

Two of the 3 dogs originally weaned to the diet each experienced occasional epileptic-type seizures during the early part of the year. Attempts are under way to determine the causes of the seizures.

In late April, dog 0271, a member of the litter that suffered from nutritional myodegeneration 2 years ago, suddenly developed paralysis of the rear limbs accompanied by urinary incontinence. A blood sample revealed no abnormality in red and white cell counts or in differential count. X-ray examination revealed the presence of bony spurs on the second, third, sixth, and seventh lumbar vertebrae and a rough, distorted area on the eleventh thoracic vertebra.

The possibility of a recurrence of vitamin E deficiency was considered. Sheep and cattle suffering from white muscle disease show greatly elevated levels of serum glutamic-oxalacetic transaminase (SGOT). Therefore, SGOT determinations were performed on blood samples from dog 0271, from one of her litter mates, and from 2 stock dogs of the same age. The SGOT values for these four dogs ranged from 20 to 46 units/ml - all within the normal range (10-50 units/ml). Thus a vitamin E deficiency appeared unlikely, and later necropsy did not reveal the characteristically pale areas of musculature.

Since the condition of the animal remained unchanged, she was killed on the fourth day after the onset of paralysis. Until the central nervous system was examined, necropsy had revealed a grossly normal, healthy dog. The spinal cord in the lumbar area was extremely congested and hemorrhagic. The brain showed marked hydrocephalus in the first and second ventricles, a condition believed to be secondary to the congested and apparently blocked spinal canal.

Subsequent X-ray examinations of the other 3 dogs of this litter showed that 2 of them had similar bony spurs in approximately the same region of the spinal column. A complete series of X-rays is now being taken on all animals raised to adulthood on the semisynthetic food.

Comparisons are being made of dogs on the semisynthetic food and those on the stock diet. This study will cover general physical condition, hematology, reproductive performance, pup mortality, analysis of growth, and calculations of the efficiency and protein efficiency of the diet.

Physical Examinations of Dogs on the Semisynthetic Diet

As of July 30, 1963, 15 beagles have received a semisynthetic diet(2) exclusively for periods ranging from 18 to 33 months. Five dietary formulations have been used and the dogs have received 3, 4, or all 5 of these variations within this time period (Table 9). One of the dogs died in April, 1963 as a result of localized hemorrhage involving the spinal cord. It could not be determined whether or not the hemorrhage resulted from traumatopathy. Another dog was not subjected to radiology because of her gravid state. Sufficient information was available, however, to warrant including all 15 dogs in one group.

TABLE 9

Schedule for 15 beagles maintained on semisynthetic diet
from dates listed to July 30, 1963

Dog number	Sex	Birth date	Born on diet	Diet variations fed*				
				CASA	CASB	CASC	CASD	CASE
0172	M	7/17/60	No	X		X	X	X
0173	M	7/17/60	No	X		X	X	X
0174	M	7/17/60	No	X		X	X	X
0271	F	2/10/61	Yes	X	X		X	
0272	F	2/10/61	Yes	X	X		X	X
0273	F	2/10/61	Yes	X	X		X	X
0275	M	2/10/61	Yes	X	X	X	X	X
0280	M	3/4/61	Yes	X	X	X	X	X
0281	M	3/4/61	Yes	X	X	X	X	X
0284	F	3/26/61	Yes		X		X	X
0285	M	3/26/61	Yes		X		X	X
0409	M	12/23/61	Yes		X		X	X
0410	M	12/23/61	Yes		X		X	X
0412	F	12/23/61	Yes		X		X	X
0413	F	12/23/61	Yes		X		X	X

*ANL formulations.

An interim examination of these adult dogs was undertaken to evaluate the effects of the semisynthetic diet, to conclude the first phase of this ancillary study. The evaluation deals essentially with the present physical state and the immediate past medical history of the dogs. They were subjected to a complete physical examination which included electrocardiography, phonocardiography and radiology.

Recent Illness. Dog 0172 has mild ideopathic epileptic events probably averaging 2 per month. Dogs 0173 and 0174 are similarly affected at intervals of 2 to 3 months. Dog 0271 exhibited bilateral paralysis of the pelvic limbs described earlier; the paralysis was accompanied by incontinence, cystitis and physical deterioration which dictated euthanasia.

Temperature, Pulse, Respiratory Rate. Values for body temperature averaged within 0.1 to 0.2°C of those obtained from comparable groups of dogs. Pulse rates and respiratory rates also appeared to fall within the ranges of values obtained from other groups of dogs.

Attitude or Temperament. This factor was noted to be unusually good in the group as a whole.

Condition or Weight. Three were considered obese. All other dogs were in acceptable to excellent condition with regard to desirability of present weight, judged upon appearance.

Hair Coat. In 8 dogs the hair coat was in good condition, in 5 it was shaggy or coarse and generally lacked lustre, and in 2 it was dry or dull in appearance. While there is abundant evidence that hair coat characteristics are strongly influenced by heritable factors, particularly length and coarseness of the hair, there is also evidence that lack of lustre is a sign of less than optimum health.

Skin. The skin of the dorsal thoracic region was examined primarily for texture and found to be normal. The skin adjacent to the lips was hyperemic, slightly edematous, sensitive, and somewhat devoid of hair.

Muscle Tone. This factor is difficult to measure since it may be influenced to a considerable extent by temperament and response to the environment. However, an attempt was made to evaluate it during the examination with the result that four dogs were judged better than average and the rest average. Significantly, in no case could muscle tone be interpreted as poor; frequent prior impressions were that the group as a whole is somewhat superior in this respect.

General Appearance or Grooming. Ordinarily grooming reflects the sort of care that animals receive but in this case it may be considered to reflect vitality. A healthy animal usually manages to maintain a well-groomed appearance, particularly in the environment which these dogs enjoy. Four dogs appeared well-groomed, 2 poorly-groomed and the others average. The character of the hair (i.e., length, coarseness) was disregarded in evaluating this condition.

Skeleton. Examination by palpation indicated that all dogs have apparently normal skeletons except 0271, in which we suspected a tumor or injury involving one or more of the caudal thoracic vertebrae.

Musculature. Muscular development appeared normal in all dogs; however, 0271 had severe muscular spasms involving the head, neck and a thoracic limb, very likely related to the spinal abnormality described in the preceding paragraph.

Lymph Nodes. Three sets of lymph nodes, the mandibular, pre-scapular, and popliteal can ordinarily be palpated on examination. In this group the nodes obviously varied considerably in size; it was not determined by comparison whether the variation was significant.

Eyes. The eyes were judged to be normal.

Ears. Almost every dog had an excessive accumulation of cerumen and primary otitis was similarly frequent. However, the two conditions did not coincide exactly so the otitis is not necessarily a result of excess accumulation of cerumen. The extremely high incidence of these two conditions has not been apparent in other groups of dogs in this colony.

Oral Cavity. These observations include an examination of the tonsils, tongue and the mucosa other than the gingivae. The abnormalities noted were undoubtedly transitory. In three cases one or both tonsils were enlarged and in two cases the pharyngeal mucosa was inflamed or hyperemic.

Gingivae. Without exception these dogs suffer from gingivitis. Most had one or a combination of the following conditions: ulcerative gingivitis, hyperplasia, atrophy, a nodular proliferation of the submucosal connective tissue; in one case the cementum of the teeth was visible. Radiology (see below) indicated where there was positive evidence of periodontal disease. These same conditions exist in animals maintained on a conventional diet.

Teeth. A moderate amount of calculus was observed in 1 dog; in all other cases the accumulation was marked. (A marked accumulation is such that it is immediately adjacent to and/or has displaced the gingivae to some extent.) The calculus observed here differs from that usually seen in dogs on a conventional diet in that the surface is rough and pitted. As a rule the calculus in these dogs is lighter in color on the molars and premolars but is often black or partially black on the incisors.

The number of teeth varied so much that it is more convenient to present the observations in tabular form (Table 10).

Failure of permanent teeth to erupt is often attributed to a hereditary factor and the persistence of the deciduous teeth to lack of dental exercise. Failure of permanent teeth to erupt has been frequently noted in this colony in the dogs on the conventional diet; persistence of the deciduous canines in the same group of dogs has been seen also but less frequently.

TABLE 10

Summary: Teeth of dogs on semisynthetic diet

Dog number	
0172	Permanent, all present
0273	"
0280	"
0281	"
0285	"
0409	"
0410	"
0412	"
0173	Missing: Third molars (mandibular).
0174	Missing: Lower right 3rd premolar
0271	Deciduous upper canines persist
0272	Deciduous upper canines persist
	Missing: Lower left 2nd premolar
0275	Missing: Both upper second molars, lower left 4th premolar, lower right 2nd premolar
0284	Missing: Lower left 3rd molar. Lower right 2nd molar definitely mobile
0413	One supernumerary or deciduous tooth present, anterior to upper right 1st premolar

Respiration. This is normal in general although a tendency to forced expiration was observed in one or more dogs. This tendency has not been noted in other dogs and, for the present, cannot be explained.

Lung Sounds. Abnormal sounds were as follows: 0271, moderate rales; 0172, 0273, and 0275, slight rhonchi. These abnormalities may be transitory and due to respiratory allergy or inhalation of particulate matter. However, all these dogs were raised and maintained on this diet longer than most of the others examined.

Cardiac Rhythm and Sounds. These were normal.

Palpation of Abdominal Organs. This portion of the examination was difficult because invariably the abdominal muscles were vigorously contracted. Although it was difficult to be certain, a number of the dogs,

one certainly, gave indications of discomfort or pain when attempts were made to palpate the abdominal organs. Dog 0281 indicated definite discomfort particularly in the renal area. Despite the uncertainties, the possibility cannot be disregarded that the abdomens of these dogs were tense because of pain or tenderness.

Hernias. None were apparent.

Mammary Tissue. This was found to be normal.

Anus. With no exceptions the anal mucosa was somewhat swollen and inflamed, and in some cases considerable discomfort was indicated during rectal palpation. Fecoliths were always present in the rectum and all of the dogs were thought to suffer chronic constipation, which may explain the abdominal tenderness and the rectal inflammation.

Male Reproductive Organs. One dog was anorchous (this characteristic is heritable). Another had a small, flaccid prostate gland.

Breeding Performance. Libido was well expressed in the males in most of the testing periods. In several instances it was necessary to utilize males maintained on conventional diet in order to impregnate the females after each of the males on this special diet was given two or more trials. However, since 2 of the males have each been responsible for two pregnancies this does not constitute a problem. The females regularly demonstrate estrus and have proven capable of successful gestation.

The absence of a comparable control group precludes any statement as to the normality or abnormality of breeding performance in this group.

Urinary Tract. Only 1 dog was abnormal; it was incontinent and had abnormal urine indicating cystitis. This was dog 0271, which was paraplegic when examined.

Electrocardiograms. Only 2 dogs provided what might be interpreted as abnormal electrocardiograms. Dog 0272 may possibly have a defect in the auricular musculature and a partial block of impulse transmission in the ventricles. It is not unusual to have indications of either of these conditions in healthy animals but rather more uncommon to see them both on the same tracing from an animal unless there is a heart defect. The phonocardiogram and the stethoscopic examinations revealed no abnormalities, and the animal's condition otherwise gives no cause for concern at the time.

The electrocardiogram from dog 0281 gave some indication of auricular fibrillation; however, this also occurs in apparently healthy dogs. The most likely explanation for the abnormal tracing is that it reflects the effects of anesthesia.

Hematology. The last hemograms for these dogs were obtained between February and May 1963 except for dogs 0409 through 0413, for which no hemograms have been obtained. In evaluating the hematocrits, hemoglobin values, total erythrocyte counts and total leukocyte counts there is little or nothing to indicate abnormal hematopoiesis. Ability to regenerate hemoglobin is not included in this evaluation since it has been covered previously.(2)

Radiology. All roentgenograms considered here were made on Kodak No-Screen film using the Philips 150-kv constant potential machine. Multiple views were taken so that all skeletal structures except the forelimbs were exposed to dorso-ventral and lateral inspection. Dental abnormalities were noted above and are not repeated here, but abnormalities of osseous supporting structure of the teeth are included. Observed abnormalities were as follows:

Dog 0174: Heart enlarged to the extent that the shadow measured 8 x 11 cm; normal for a dog of this size, 7 x 9 cm.

Dog 0271: Apparent faulty development of the dorsal process of T5* was shown by the enlarged and irregular outline of the caudal border. Exostoses of vertebrae were apparent in both lateral and ventro-dorsal views. Spurs extended ventrally and right latero-posteriorly from L6 and anteriorly from L7.

Dog 0272: Obvious exostoses appeared as spurs both anterior and posterior from T11, anterior from T12, anterior and bilateral from L6, anterior and posterior and bilateral from L5, posterior and bilateral from L4. There was vertical bone loss between molars 1 and 2 in the lower left quadrant.

Dog 0275: The costal cartilage of one T6 rib appeared deformed and that of one T7 rib was probably fractured. The heart was enlarged (shadow 7.5 x 11 cm). Exostoses were visible at the L6 and L7 intervertebral space level with both ventral and bilateral growth.

Dog 0280: A fracture with a well-formed callus involved the dorsal process of T7. The proximal tibiae were observed to have exaggerated medial convex configuration.

*Abbreviations used here are C (cervical vertebra), T (thoracic vertebra) and L (lumbar vertebra).

Dog 0281: There was exaggerated medial convex configuration of the proximal tibiae.

Dog 0284: Exostoses, all extended ventrally, were seen on C6, T1, T11, T12, T13, L1, L2, L3, L4, L5, L6, and L7. Exostoses in L3, L4, L5, L6, and L7 extended laterally as well. The fibulae appeared abnormally convex; the impression was that this was both posterior and medial. The tibiae may also be slightly distorted. Definite coarsening was apparent in the trabeculae supporting the teeth, and the inner cortical border of the mandible was irregular.

Dog 0285: There was a partially healed fracture at the juncture of the middle and distal one thirds of the right fibula. Vertical bone loss due to periodontitis involved all three molars of the left mandible.

Dog 0409: A sclerotic area 0.5 cm long was present in the proximal right fourth rib.

Dog 0410: An exostosis extended antero-ventrally from the body of T3.

Dog 0412: The humeri appeared to have an abnormal or exaggerated curvature in which there was some coarsening of the trabeculae.

Summary

1. Symptoms of illness have been reported recently which could be due to heritable defects, but these same defects could be aggravated by environmental and dietary effects.
2. Three dogs were obese and the rest within an optimum weight range as judged by appearance.
3. In nearly half of the dogs the hair lacked the lustre and texture observed in well-nourished dogs.
4. Muscle tone was above average in comparison with the colony as a whole.
5. Almost every dog had primary otitis externis and excess cerumen in the external auditory canal.
6. Congenital absence of teeth occurred with about the same frequency as in the colony as a whole. The same is probably true of persistent deciduous teeth.

7. In some of the dogs the abdomen seemed abnormally sensitive, possibly as the result of chronic constipation.
8. In all of the dogs the anal mucosa was swollen and inflamed, probably partially or entirely due to chronic constipation.
9. Cardiography exposed possible heart defects in two dogs although there was no evidence of functional embarrassment. Two dogs had enlarged hearts.
10. The incidence of vertebral exostosis was high in comparison with 25 dogs on standard diet; 22 vertebrae being affected in these 15 dogs in contrast to 2 vertebrae having exostoses in the 25 controls. Fractures were apparent in two dogs and evidence of fatigue fractures in another.
11. The exaggerated convex configuration of the tibiae seen in three dogs may be "normal," i.e., a heritable characteristic; however, a similar configuration of fibulae has not been viewed previously in several hundred examinations.

Conclusions

The semisynthetic diet has proven capable of supporting growth and maintaining weight in unselected beagle stock. Muscle tone is above average and general health has been equivalent in comparison to that of dogs fed a standard diet. The incidence of vertebral exostoses is higher than the average for dogs of equivalent age fed on a standard diet. Bone fractures have not been recorded (radiographs), but certain of the variations in bone formations have been noted previously in control dog populations. The constipation and rectal irritation can be eliminated through changes in food formulation.

It appears that the results to date justify continuation of this nutritional study. In programs like this one, in which nutritional factors may be either experimental variables or potential sources of unwanted variation, it would be more convenient and less expensive to use a semisynthetic diet whose composition can be controlled with relative ease.

Effect of Co⁶⁰ γ Radiation upon Urinary Reducing Substances*

This study concerns the identification of a urinary reducing substance excreted by dogs after Co⁶⁰ γ -radiation of 325 r, and originally believed to be glucose. Subsequent testing failed to confirm this belief but suggested the substance might be glucuronic acid or one of its conjugates. Several more dogs were irradiated, and the original two dogs

*This work was performed by Mrs. Lillian V. Kaspar.

were re-irradiated,(1,2) but it was not possible to reproduce the original results. After these several attempts had failed, the study of the elusive reducing substance was abandoned.

In addition to the chemical determinations previously described, blood samples from dogs 0422 and 0424 were assayed for serum lactic dehydrogenase (average values 200-600 units/ml/min) and serum glutamic-oxalacetic transaminase (average values 10-50 units/ml/min). No deviations from the normal ranges were encountered.

Amino Acid Analysis*

A number of reports in the literature describe changes in free amino acids of blood and urine after exposure to ionizing radiation. Such reports have prompted the initiation of a preliminary study of free amino acids in the blood of beagle dogs. Because of limited equipment at our disposal, only a few of the amino acids can be studied comprehensively; the basic amino acids and proline and hydroxyproline have been selected.

Although the ratios of amino acids in fasting blood samples exhibit only small individual variations, blood levels of amino acids are greatly influenced by the amino acid composition of dietary protein. All analyses must be done on samples from fasting dogs, and the amino acid composition of diet should be periodically checked.

Proline and hydroxyproline have previously been separated in 0.9 x 50 cm columns of Dowex 50 (chloride form) by elution with 1 N HCl, and this procedure will be followed here. The basic amino acids will be separated on a 0.9 x 15 cm column of Dowex 50 (Na⁺ form) by a series of buffers; a few preliminary runs followed by paper chromatography showed that separation can be achieved.

The following aspects of the problems involved in such a study have been partially investigated: 1) The development of a satisfactory method for the separation of amino acids from blood proteins, salts, sugars, etc. Methods under consideration are picric acid or tungstic acid precipitation, adsorption of amino acids on Zeo Carb 225 followed by subsequent elution, or microdialysis. 2) Construction and calibration of a suitable 1-ml siphon to be used in conjunction with the fraction collector. 3) A study of the ninhydrin and modified ninhydrin methods for amino acid analysis. After one of the methods has been chosen, calibration curves must be run on the appropriate amino acids.

*This work was performed by Mrs. Lillian V. Kaspar.

Summary of Findings on Living Dogs with Ce¹⁴⁴ Burdens

All the dogs with Ce¹⁴⁴ burdens that are still alive were given a physical examination during this period. Since there seem to be no differences in symptoms or signs between the 50 $\mu\text{C}/\text{kg}$ and 25 $\mu\text{C}/\text{kg}$ groups, they will be considered as one.

No one abnormal condition attributable to irradiation is common to all 14 of these dogs. Five had some excess loose hair in the pelt and/or had dry lusterless hair. Ordinarily three sets of lymph glands can be palpated in beagles, but this rather small number of dogs includes two in which one gland was not palpable and one in which none of the 6 glands was palpable; in general the glands were smaller than normal, but in some they were of average size. Considering the age of these dogs, it seems almost abnormal that not one is obese.

Certain conditions existed in one or more dogs that were certainly not due to radiation damage: prostate glands were enlarged in four and the epididymis in two. One had a significant heart murmur and another a minor and intermittent heart murmur.

The only condition observed in all 14 dogs is excessive dental calculus formation, accompanied nearly always by minor to irreversible soft tissue damage. There is undoubtedly some periodontal damage as well, but it is impossible to assess the extent of this without removing the calculus and obtaining dental roentgenograms.

Three of the dogs gave indications of discomfort when the kidneys were palpated and a like number had slightly inflamed anal glands. These, like other conditions that affected only one or two animals, do not appear to be significant.

Electrocardiographic tracings have been made for all 14 dogs, once in August, 1962 when the dogs were anesthetized with Pentothal sodium and again in April, 1963, without anesthesia. For earlier tracings the dogs were in the right lateral recumbent position, while for the later ones they were standing.

Conventional measurements were made on the lead II tracings and the averages for the two different positions were compared (Table 11). Although the two sets of tracings were taken at two different times the average values are remarkably similar. It would seem then that positioning is not important; however, tracings from anesthetized dogs were superior because of the absence of skeletal muscle tremors. The standing position, however, has one major advantage: the tracings can be obtained more frequently and possibly with less risk to the dog. Further examination and appraisal of these data (see Table 11) is not warranted because they were taken at different times and because relatively few dogs are represented.

TABLE II

Comparison of average values in cm obtained from lead II
electrocardiographic tracings from 14 living dogs
with Ce¹⁴⁴ burdens

	Anesthesia, right lateral recumbency*	No anesthesia, standing position*
P-Wave interval	0.03625	0.03865
P-Wave amplitude	+0.21	+0.204
P-Q interval	0.10009	0.10354
Q-R-S interval	0.0382	0.0365
Q-Wave amplitude	-0.3786	-0.5007
R-Wave amplitude	+1.926	+1.934
S-Wave amplitude	-0.3929	-0.3036
S-T interval	0.2016	0.1825
T-Wave interval	0.0933	0.07436
T-Wave amplitude	+0.138 (6)	+0.1522 (9)
	-0.21 (8)	-0.184 (5)
S-S interval	0.5511	0.6185

*+, positive deflection; -, negative deflection.

All intervals and amplitudes are given in centimeters. For conversion of intervals each cm equals 0.13 seconds; each cm amplitude equals 1 millivolt.

Cooperative Cardiac Studies. Dorsal ventral and lateral roentgenographic views of the thorax taken from more than 50 different beagles in our colony were examined by Dr. Robert Hamlin (Ohio State University, Columbus) in a study of the size of the beagle heart and its relative position in the thorax. This first cooperative study may be extended into a long-term investigation of cardiac conditions in this colony.

Parasitology. Approximately 18% of the dogs have evidence of parasitism; this incidence is unchanged since the previous reporting period.

Hematology. The task of maintaining even a minimum program has long since become impossible as the size of the colony has increased. A total of 536 hemograms was obtained from the dog colony during this reporting period, but only a few included differential leucocyte counts.

A correction formula for the Coulter counter has been derived which may be readily adapted to any aperture tube on any Coulter instrument.

The derivation involved determination of the weighted means of the resolving time of the instrument. The operation required many machine operations and lengthy computations; however, at this point, it is possible to reproduce any or all of the data and computations as required. A complete IBM hematology program was then written to correct all previous data and all future data obtained in this fashion. The program also includes a section for retrieval of basic computed data. Operations are now in process by the IBM installation to retrieve all data taken to this time in common form.

In addition, some time has been devoted to the programming details for the future use of the Coulter sizing procedure in conjunction with a multichannel analyzer and the IBM 536 keypunch. The final details will have to be worked out after installation of the new equipment although all of the input and output card layouts and descriptions have been tentatively completed.

Dental Studies. A considerable amount of time has been given to a study and discussion of the dental problems in this dog colony. An evaluation of the problem and a program proposal to alleviate the problem has been written and submitted for consideration.

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DOSE TO GONADS FROM INJECTED Sr^{90} AND Y^{90}

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It has recently been suggested that genetic effects of injected Sr^{90} may result in part from fixation of the strontium isotope in close connection with the DNA molecule.(1) This suggestion has been based on experiments in which an intraperitoneally injected Sr^{90} - Y^{90} equilibrium mixture was more effective (in terms of microcuries injected per gram of body weight) than Cs^{137} in reducing the viability of uterine implants in the mouse after either the male or the female parent had been injected with the radionuclide.

In order to evaluate such an experiment, it is necessary to estimate the gonadal dose that results from the injection. This dose may be acquired either from radionuclides present in the soft tissue of the gonad in the early period when the nuclide is circulating, before bone deposition or excretion, or by irradiation from nearby bone in which deposition has taken place. If the degree of genetic damage (reflected through dominant lethal mutations, in failure of implant development) were to exceed that anticipated to result from irradiation of gonadal tissue, it might be suspected that transmutation and recoil of Sr^{90} in the DNA molecule play an important part in the result.

In these experiments, male CF #1/Anl mice 2-4 months of age were injected intraperitoneally with $5 \mu\text{c/g}$ of a carrier-free equilibrium mixture of Sr^{90} and Y^{90} . At suitable intervals the mice were sacrificed by bleeding from the heart, and samples of whole blood, testis, and (in some cases) bone were analyzed for radioactivity. The samples were digested with fuming HNO_3 and heat and were counted by an end-window Geiger-Mueller counter with and without a 215 mg/cm^2 aluminum absorber. From these counts, made at various time intervals, we computed the Sr^{90} and Y^{90} present at the time of sacrifice. Counts after storage for about two weeks were made in order to correct for the self-absorption of the weaker Sr^{90} beta ray, since after that interval the samples could be assumed to contain (within 2%) equal microcurie amounts of the two nuclides.

Standard errors in the computed quantities of Sr^{90} and Y^{90} were calculated not to exceed 3% except where the values were very low. Since the blood samples were measured volumetrically in tuberculin syringes, they were subject to a sampling error of 10 or 15%. Errors in weighing the testes were less than 1% and those in weighing bone samples (taken from the femora) depended on the amount of soft tissue weighed with the bone specimens. Results of the measurements are shown in Table 12.

TABLE 12

Relative activity of mouse tissues ($\mu\text{c/g}$ tissue $\div$ injected $\mu\text{c/g}$ mouse)

Time	Heart blood		Left testis		Femur	
	Sr ⁹⁰	Y ⁹⁰	Sr ⁹⁰	Y ⁹⁰	Sr ⁹⁰	Y ⁹⁰
30 min	0.58	0.58	0.18	0.36		
100 min	0.44	0.44	0.10	0.20		
200 min	0.21	0.15	0.098	0.17	2.98	2.34
	0.18	0.11	0.091	0.27	1.58	1.60
500 min	0.092	0.062	0.059	0.063	1.86	1.58
	0.095	0.036	0.077	0.170	2.58	2.08
21 hr	0.027	0.011	0.032	0.049		
	0.028	0.009	0.042	0.660		
45 hr	0.009	0.004	0.016	0.046		
	0.010	0.007	0.017	0.057		
	-	-	0.014*	0.033*		

*Right testis

While this experiment is preliminary in nature and involves a small number of observations, it provides certain clear indications that bear on the question of dosage to the gonads: 1) for the first two or three hours, yttrium and strontium seem to be cleared from the blood about equally, while after that time, the clearance of yttrium is somewhat greater; 2) Y⁹⁰ activity in the testis is subject to extreme variations under the conditions of injection, and throughout the first two days its activity exceeds that of Sr⁹⁰; 3) as early as three to four hours, the specific activity of bone (femur) with respect to both nuclides is a factor of 10 or more higher than that of the testis.

Estimation of radiation dosage to germinal tissue is extremely difficult since it comes from several sources: Sr⁹⁰ within soft tissue; Y⁹⁰ deposited in soft tissue; Y⁹⁰ from the decay of Sr⁹⁰ in soft tissue; and irradiation of gonads and genital tract from Sr⁹⁰ and Y⁹⁰ in the pelvic and vertebral bones and the urinary tract.

Y⁹⁰ will be the major factor in tissue dosage because its mean beta-ray energy exceeds that of Sr⁹⁰ by a factor of about 4.5, and, in the case of irradiation from neighboring structures, because of its greater range. In Table 13 are given dose rates calculated from means of the values in Table 12, as they would be in a large volume of the tissue in question at the concentrations found. For comparison, we have computed dose rates

from Cs^{137} in the same way, using the total-body retention values reported by Devik and Brues.(2) The practical impossibility of estimating a mean testicular dose, due chiefly to the presence of sporadic high retention values, is obvious. Estimation of external dosage, as from Y^{90} in the skeleton, is also impossible, owing mainly to uncertainties concerning variations in position of the testes. It might be mentioned that the radiation dose at a distance of 3 mm (in Lucite) from a clinical Sr^{90} - Y^{90} applicator is about 20% of that at the surface,(3) and that the dose rate at a distance of 3 mm in tissue from a $0.4 \mu\text{c}$ point source (corrected to 200 mg of the bone at the concentrations measured) is about 1 rad/hr.

TABLE 13

Rad/day per $\mu\text{c/g}$ injected, from individual isotopes (computed on the basis of dosage within a large mass of uniform composition). For Y^{90} in testis, high aberrant values are omitted in the first column and included in the second.

Time	Blood		Testis			Bone		Whole body
	Sr ⁹⁰	Y ⁹⁰	Sr ⁹⁰	Y ⁹⁰ (low)	Y ⁹⁰ (mean)	Sr ⁹⁰	Y ⁹⁰	
30 min	7.0	32.0	2.2	20.0	20.0	-	-	-
100 min	5.4	24.2	1.2	11.0	11.0	-	-	-
200 min	2.4	7.2	1.1	9.4	12.2	27.4	108.2	-
500 min	1.1	2.7	0.82	3.5	6.4	26.8	101.0	-
21 hr	0.33	0.55	0.43	2.7	19.5	-	-	9.6
45 hr	0.11	0.30	0.19	2.4	2.4	-	-	7.4

Glass microdosimeters are being calibrated for Sr^{90} - Y^{90} in order to make direct measurements of beta-ray dose rates within the animal. One mouse was sacrificed two days after injection, and dose rate measurements were made on autoradiographs by Dr. J. Marshall (Radiological Physics Division) over the intact anterior surface of the frozen carcass, using calibrated films. The maximum dose rates (corrected to 1 $\mu\text{c/g}$ injected) were seen over the pelvic bone (14 rads/day) and over the knee joints (28 rads/day).

The soft-tissue distribution of injected Y^{90} may vary according to the nature of the injection solution. Graul and Hundeshagen(4) have emphasized this, noting that the ovary and, to a smaller extent, the testis accumulate higher concentrations than most of the soft tissues, and that this effect is most marked when a small amount of yttrium carrier is used. The

differences in concentration seen in testes of our mice might be explained by occasional leakage of the intraperitoneally administered fluid into the scrotal sac. Nilsson and Ullberg⁽⁵⁾ noticed accumulation of intraperitoneally injected Sr^{90} - Y^{90} in the inguinal ring.

In considering extrapolations of mouse data to large animals, two matters should be kept in mind. The human gonads lie much farther from skeletal structures than do those of the mouse; except perhaps in the case of an emaciated female, the gonads are probably beyond the range of the Y^{90} beta ray. On the other hand, one might expect longer retention in the circulating blood and soft tissues in larger animals. This is borne out by comparisons of blood levels of Sr^{90} in the mouse with those described by Stover and Atherton⁽⁶⁾ in the dog. In the two days after intravenous injection, the blood concentration values, adjusted on the basis of $\mu\text{c}/\text{kg}$ injected, are about an order of magnitude higher than in the mouse. Blood levels of Sr^{89} measured by Norris and Kisieleski⁽⁷⁾ in the 250 g rat are quite similar to those found in the mouse. Insofar as strontium in soft tissue is freely exchangeable with that in blood, comparable differences would exist in the total radiation dose delivered from that source.⁽⁸⁾

In view of the foregoing observations, it would appear impractical to attempt a precise estimation of gonadal dose in the mouse from intraperitoneally injected Sr^{90} - Y^{90} . We plan to carry out further studies in conjunction with studies of cytological changes in blood and bone marrow.

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EFFECTS OF PLUTONIUM INJECTED SUBCUTANEOUSLY IN THE RAT AND MOUSE

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A previous report(1) dealt with effects of single and multiple subcutaneous injections of methylcholanthrene. The late carcinogenic effects of similar injections of Pu^{239} are reported here.

Materials and Methods

All experimental animals received 0.05 ml injections of isotonic saline containing $0.98 \mu\text{g Pu}^{239}$ and $0.0018 \mu\text{c Ce}^{144}$ (the latter as a gamma tracer for external counting). The original solution contained 1 mg $\text{Pu}^{239}/\text{ml}$ in 1 M HNO_3 . Of this solution, 0.1 ml was diluted to 5 ml with isotonic saline. A tracer solution of Ce^{144} ($18.36 \mu\text{c}/\text{ml}$) was prepared and diluted to $1.836 \mu\text{c}/\text{ml}$ in $\text{N}/10 \text{ HCl}$ and 0.1 ml of this Ce^{144} solution was added to the 5 ml of Pu^{239} solution. The pH of the injected solution was about 2. Control animals were injected with 0.05 ml of $\text{N}/50 \text{ HNO}_3$ in saline.

Rats received eight injections subcutaneously, four in each flank about 2 to 2.5 cm apart. Mice received one injection subcutaneously in the back.

Gamma counts of each mouse in the CF No. 1 group were made to determine if a significant portion of the solution had been lost. No indication of loss was found, nor was there a significant difference in gamma counts from cage to cage.

Thirty-three Wistar (WPF) female rats, 38 Sprague-Dawley (SD) female rats, 59 CF No. 1 female mice and 41 C57BL female mice were treated according to the following schedule:

Strain	No. injections	Age, days	No. animals	
			Experimental	Control
<u>Rats</u>				
WPF	8	91	23	10
SD	8	116	29	9
<u>Mice</u>				
CF No. 1	1	71	40	19
C57BL	1	79	29	12

Both rats and mice were confined no more than six to a cage and maintained on commercial chow and water ad libitum. All animals were checked weekly for palpable tumors, beginning at the time of injection. Masses were first recorded when they were 5 mm in diameter, and measurements of many of them were made every two weeks thereafter.

All animals were kept until death, at which time tumors and grossly abnormal tissues were removed and prepared for histological examination. Whole skeletal X-rays were taken at necropsy to ascertain whether bone tumors were present. Where bone tumor was suspected, sections were removed and prepared for histological examination.

Results

The results of this experiment indicate that some of the plutonium was sufficiently soluble to have been absorbed and deposited in the bone. Tumor production is shown in Table 14. Forty-four per cent of the WPF and 17% of the SD rats injected with plutonium had osteogenic sarcomas. Some of the plutonium remained at the site of injection and produced tumors there. Thirty-nine per cent of the WPF and 31% of the SD rats showed malignancies of the connective tissue or mammary glands identified as sarcoma, reticulum cell sarcoma, fibrosarcoma or mammary

TABLE 14

Summary of tumor development in rats

	WPF		SD	
	Experimental		Control	
	No.	% of total	No.	% of total
<u>Single Tumors</u>				
Osteogenic sarcoma	5	21.7	0	13.8
Other malignancy	3	13.0	0	27.6
Benign mammary	4	17.4	4	34.5
Total	12	52.1	4	75.9
<u>Multiple Tumors</u>				
Osteogenic and other malignancy	3	13.0	0	0
Osteogenic and benign mammary	1	4.4	0	3.4
Osteogenic, benign, and other malignancy	1	4.4	0	0
Other malignancy and benign	1	4.4	0	3.4
Multiple other malignancies	1	4.4	0	0
Multiple benign	0	0	2	6.9
Total	7	30.5	4	13.8
Died without tumor	4	17.4	6	10.3
Total	23	100.0	10	100.0
			29	9

adenocarcinoma. None of the control animals developed malignant tumors. In the experimental groups, 17% of the WPF rats and 41% of the SD rats developed only benign mammary tumors, and 17% of WPF and 10% of SD rats died with no tumor. Thus, 65% of WPF and 50% of SD rats died of osteogenic sarcoma or other malignancies.

Multiple tumors occurred in 30% of the WPF and 14% of the SD rats. The lower incidence of multiple tumors in the SD rats may be attributable to the lower incidence of osteogenic sarcoma in these animals, since 70% of the WPF rats showing multiple tumors had osteogenic sarcoma in conjunction with another tumor.

Table 15 shows the distribution of tumors by type among the rats. In the experimental rats, although there were 8 injection sites, an average of only slightly more than one tumor per animal was developed. The probability of developing multiple tumors is qualified by the speed with which the first tumor kills the animal.

TABLE 15
Total tumors (rats)

	WPF		SD	
	Experimental	Control	Experimental	Control
Osteogenic sarcoma	10	0	5	0
Non-osteogenic sarcoma	5	0	5	0
Mammary adenocarcinoma	7	0	4	0
Benign mammary tumor	7	4	17	11
Total tumors	29	4	31	11
Animals with tumor	19*	4	26**	8
Animals without tumor	4	6	3	1
Total animals	23	10	29	9

*7 animals with multiple tumors, 12 with single tumors

**4 animals with multiple tumors, 22 with single tumors

The average days to death by type of tumor are shown in Table 16. Of animals dying with tumor, those with osteogenic sarcoma showed the

TABLE 16
Average days to death by type of tumor (rats)

Type of tumor	SD		WPF	
	Experimental	Control	Experimental	Control
Osteogenic sarcoma	480	-	553	-
Other malignant	356	-	464	-
Benign mammary	451	684	514	636
Died without tumor	295	140	426	714

longest average days to death and those dying with benign mammary tumors lived next longest. The shortest average days to death was shown by those dying of other malignant tumors. One peculiarity of the Pu injection is indicated by the result that the experimental rats dying with benign mammary tumors lived a shorter time than the controls dying with benign mammary tumors.

Of the 69 experimental mice, a total of 30 died with osteogenic sarcoma and 5 died with other tumors (Table 17). As will be seen in the table, the number of tumors that might be attributed to local effects of plutonium was not high enough to be considered significant.

TABLE 17

Total tumors (mice)

Type of Tumor	CF No. 1			C57BL		
	Experimental	%	Control	%	Experimental	%
Osteogenic sarcoma	19	47.5	0	0	11	37.9
Other malignancy	3	7.5	3**	15.8	2†	6.9
Died without tumor	18	45.0	16	84.2	16	55.2
Total	40	100.0	19	100.0	29	100.0

*1 reticulum cell sarcoma; 2 adenocarcinoma

**1 sarcoma, type unknown; 1 adenocarcinoma; 1 squamous cell carcinoma

†1 lung tumor; 1 papilloma

A comparable incidence of bone tumors has been reported after intravenous injection of a similar dose ($3.1 \mu\text{c/kg}$) in CF No. 1 mice.(2) An earlier experiment, in which 30 mice were given single subcutaneous injections of $1 \mu\text{g}$ plutonium, had yielded 8 osteogenic sarcomas and 4 subcutaneous sarcomas, the latter near the injection site.(3)

Conclusions

Plutonium, injected subcutaneously in WPF and SD rats, produced malignant tumors at the injection site and also in the skeleton. Subcutaneous injection in CF No. 1 and C57 BL mice yielded malignant tumors in the skeleton, but no locally induced malignant tumors were found in 60 animals so injected. The solubility of the plutonium solution and its transport to bone may have decreased the total possible production of malignancies at the injection site. An experiment to verify this, by the use of particulate or colloidal plutonium suspensions, is in progress.

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ATTEMPTS AT REDUCTION OF ERYTHROCYTE CATALASE LEVEL. II.

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The desirability of reducing the erythrocyte catalase level in oncological studies has been discussed earlier.(1) 3-Amino-1,2,4-triazole (AT) has been shown to be effective in reducing the catalase of all tissues but that of the erythrocyte,(2) and preliminary attempts to reduce the erythrocyte catalase activity by several other techniques have been reported.(3) We have also reported some striking hematological effects induced by injection of the H_2O_2 -producing enzyme glucose oxidase.(4) Elsewhere in this report(5) is given a description of effects on catalase of a wide variety of compounds related to AT, none of which, however, has by itself any effect on erythrocyte catalase. The present paper describes how, by combining several of the above devices, we have been able to demonstrate reduction of erythrocyte catalase activity. The effects obtained thus far are significant, though not of the magnitude we wish ultimately to obtain. Further, they are achieved under lethal or nearly lethal circumstances and are, therefore, impractical as a means of maintaining animals in an acatalasemic condition.

Methods and Materials

The animals used were female CF No. 1 and Charles River mice, usually 6-8 weeks of age. Although blood catalase in the Carworth Farm mice is on the average slightly higher than in the Charles River mice (155 perborate units(6) per ml versus 135), the variability is approximately the same, and it made little difference which strain was used although for any one experiment all mice were, of course, of the same strain and age.

All injections were intraperitoneal, and all were adjusted to 0.01 ml/g body weight. Insoluble materials were suspended in 5% aqueous gum arabic. Acid or alkaline materials were adjusted to approximately pH 7. Control values were obtained from mice correspondingly injected with saline or gum arabic.

Each datum represents 3 to 5 mice. Blood was drawn from the orbital sinus,(7) laked in an appropriate volume of cold water, and assayed for catalase by the perborate method.(6) Total hematin pigment was determined by the method of Hunter.(8)

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Results

Three general types of compounds were injected in combination:

a) glucose oxidase, as a continuing source of H_2O_2 in situ; b) AT or 5-mercapto-1,2,3,4-tetrazole, which have been shown(5) to be effective inhibitors of liver catalase in vivo and of crystalline catalase in vitro; and c) various agents usually considered to react with sulphydryl groups ("sulphydryl reagents").

From a large mass of data, the following general results may be described:

1. The injection of glucose oxidase alone, in sublethal doses, is without effect on blood catalase, but in supralethal doses will cause changes in the catalase activity per ml whole blood. However, it has been possible in all cases to demonstrate clearly that such changes parallel hematological changes observable in animals treated with glucose oxidase.(4) Thus, animals given less than about 10 lethal doses of the enzyme show an apparent increase in catalase activity; simultaneously the hematocrit rises sharply. Since the catalase is in the erythrocyte, the latter observation accounts for the former. If, on the other hand, mice are given about 20 lethal doses of glucose oxidase, considerable cell lysis is observed, pigment (including catalase) appears in the plasma, and pigment is seen to be lost in urine and accumulated in tissues. As a direct result, blood catalase activity progressively decreases; over 80% loss has been seen before death ensues.

2. If injection of AT precedes injection of lethal doses of glucose oxidase, blood catalase activity is decreased, and this is a genuine inhibition. Whether catalase activity is based on cells, volume, or pigment, an inhibition is demonstrated. This is true, however, only after lethal doses of glucose oxidase; AT preceding nonlethal doses of glucose oxidase is without effect. Typical examples illustrating the above statements are found in Table 18. (The LD_{50} of the glucose oxidase employed is about 14 mg/kg.)

3. A variety of "sulphydryl reagents" has been tested, in combination with AT and with or without added glucose oxidase. All reagents were administered in sublethal doses, although the combination of sublethal AT plus sublethal glucose oxidase is sometimes lethal. None of the sulphydryl reagents tested in combination with AT and glucose oxidase significantly affected the erythrocyte catalase. The agents tested were: N-ethylmaleimide, p-chloromercuribenzoate, 1,4-naphthoquinone, 2,3-butanedione, methyl sulfoxide, maleic acid, ferricyanide, glyceraldehyde, iodoacetic acid, iodoacetamide, porphyrindin, streptomycin, and 2,6-dichlorophenolindophenol.

4. 5-Mercapto-1,2,3,4-tetrazole, which has been shown(4) to be a significantly more potent catalase inhibitor than is AT, if administered

with glucose oxidase will reduce erythrocyte catalase. Without glucose oxidase, the mercaptotetrazole is without effect on blood catalase activity. Table 19 demonstrates that with the combined treatment, it is possible to reduce blood catalase significantly under conditions of borderline lethality.

TABLE 18

Effect of aminotriazole (AT) and glucose oxidase on blood catalase

Each datum represents average blood catalase of 3 mice. Negative figures represent decreased activity; positive figures represent increased activity. LD₅₀ for the glucose oxidase preparation used was 14 mg/kg.

Expt.	Concn. of AT, mg/kg	Concn. of glucose oxidase, mg/kg	Time, hr	Effect on blood catalase, %
1	0	100	1	+31
	1000	100	1	-75
2	0	250	1	-43
	1000	100	1.5	-87
4	1000	5	1	+4
	1000	100	1	-33
5	1000	100	2	-92
	1000	25	1	+2
	1000	25	2	-36
	1000	25	3	-34
	1000	5	3	+6
	1000	5	6	+7
	1000	100	1	-33
	1000	100	2	-92

TABLE 19

Effect of 5-mercapto-1,2,3,4-tetrazole (5-MT) and glucose oxidase on blood catalase activity

Each datum represents average blood catalase of 3 mice. Negative figures represent decreased activity; positive figures represent increased activity. LD₅₀ for the glucose oxidase preparation used was 14 mg/kg.

Expt.	Concn. of 5-MT, mg/kg	Concn. of glucose oxidase, mg/kg	Time, hr	Effect on blood catalase, %	Toxicity, dead/total
1	500	100	1	-56	
	500	20	1	-49	
	500	4	1	-3	
2	1000	10	0.5	-46	
	1000	10	1	-53	
	1000	10	2	-35	
3	1000	20	1	-40	4/4
	1000	10	1	-21	0/4
	1000	5	1	-29	1/4
	500	20	1	-49	4/4
	500	10	1	-7	3/4
	500	5	1	±0	1/4
	250	20	1	-6	4/4
	250	10	1	-15	0/4
	250	5	1	-10	0/4
	1000	20	1	-40	4/4
	1000	10	1	-21	0/4
	1000	5	1	-29	1/4

Discussion

A variety of sulphydryl reagents was included in the testing because of a suggestion by Margoliash and Schejter.⁽⁹⁾ These authors proposed that AT inhibits liver catalase but not erythrocyte catalase because the erythrocyte contains large amounts of a dialyzable, probably sulphydryl, catalase donor, and the AT is unable to compete successfully with the peroxidatic reaction of catalase with the donor. We thus attempted to alkylate, oxidize, or otherwise remove from activity at least a portion of these donor substances. Apparently this idea is not valid; mercaptotetrazole plus glucose oxidase is quite as effective in the absence as in the presence of the sulphydryl reagents tested in our system.

A question of interest is whether, in those cases in which AT plus glucose oxidase caused an inhibition of catalase, the inhibition involved only that catalase which had appeared in the plasma, or whether inhibition occurred also within the erythrocyte, which would in turn indicate that the AT had penetrated the cell. In several experiments, erythrocytes and plasma were centrifuged and separately assayed for catalase and for total hematin pigment. When only glucose oxidase was injected, the decrease in erythrocyte catalase was almost exactly proportional to the decrease in total hematin pigment of the erythrocytes, despite the fact that appreciable catalase activity and hematin pigment were lost from the cells and found in the plasma. On the other hand, if an injection of AT was followed by glucose oxidase (in a lower dose, because of augmentation of its toxicity by AT), the cells lost only about 2% of their pigment, but about 87% of their catalase. It thus seems fairly conclusive that under these conditions the erythrocyte membrane is permeable to AT. However, the drastic effects of glucose oxidase on the erythrocyte may involve an altered permeability of the membrane, so that the present results should not be used as a demonstration that AT can enter normal erythrocytes.

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EFFECT ON CATALASE IN VIVO AND IN VITRO OF COMPOUNDS RELATED TO AMINOTRIAZOLE

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Many papers have confirmed and extended the original observation by Heim et al.⁽¹⁾ that 3-amino-1,2,4-triazole (AT), upon injection into rats, reduced the catalase activity of the liver and kidney. Margoliash et al.⁽²⁾ examined the structural requirements of catalase inhibitors of the AT type, and on the basis of the compounds available to them, formulated certain minimal structural requirements for catalase inhibition.

We have been fortunate in having made available to us a large series of compounds related to aminotriazole, and others have been synthesized locally, or purchased. Most of these we have tested not only in vitro, but also in vivo. We have been able to extend and modify the conclusions of Margoliash et al.⁽²⁾ Several agents are as effective or more effective than AT. However, as was initially reported for AT⁽¹⁾ not even the most potent of these compounds by itself affects the catalase of the erythrocytes in vivo.

Materials and Methods

The following chemicals were purchased commercially: 1,2,4-triazole, 3,5-diketodihydro-1,2,4-triazole (Urazole), pentamethylene tetrazole (Metrazole), 5-aminoindazole, 6-aminoindazole, 7-aminoindazole, semicarbazide, thiodemicarbazide, isonicotinic acid hydrazide, and aminoguanidine. The following, with references to the methods of synthesis, were prepared locally: 3-acetylamino-1,2,4-triazole, (3)* 3-furfuryliden-, 3-benzyliden-, and 3-butylidenamino-1,2,4-triazole, (4) glucose, xylose, mannose, and ribose adducts of 3-amino-1,2,4-triazole, (5) and S-methylisothiosemicarbazide. (6) The 3-amino-1,2,4-triazole (AT) used was the kind gift of Dr. Stanley R. McLane, Amchem Products. All other compounds were provided by Dr. Cameron Ainsworth, Eli Lilly and Company.**

All catalase assays were performed by the perborate method.⁽⁷⁾ In vitro testing was done in a system similar to the type a system of Margoliash et al.,⁽²⁾ in which the H₂O₂ necessary for conversion of

*We wish to thank Dr. Norman Frigerio, Argonne National Laboratory, for the synthesis of the 3-acetylamino-1,2,4-triazole and for many helpful discussions.

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catalase to catalase- H_2O_2 complex I is produced by the autoxidation of ascorbic acid, 10^{-3} M in the final mixture. The system also contains phosphate buffer, pH 6.8, at 0.03 M final concentration, catalase (a commercial, twice recrystallized beef liver product, used at a concentration arbitrarily selected to give 200 perborate units* per ml final mixture, and the inhibitor to be tested, at the final concentrations shown in the tables. The mixture was incubated for 1 hr at 37°C , then diluted with ice water and assayed for catalase; the activity was compared with that of a similarly treated mixture in which water replaced the inhibitor. The technique of incubating in high concentrations, then diluting, is necessitated by the combination of some of these inhibitors with catalase to form a reversibly inhibited form of the enzyme. Since the dilution technique, however, requires considerable extra effort, glassware, etc., in the interests of time all in vitro tests were done first with a catalase solution of low enough activity to permit direct assay. If inhibition was demonstrated, then the dilution technique, as described, was used to confirm that this was a genuine irreversible inhibition. Data in the table are thus generally from use of the simpler technique.

In the in vivo tests the material was injected intraperitoneally, in amounts based on a preliminary toxicity test. Insoluble materials were injected as a suspension in 5% gum arabic. Extremely acid or alkaline materials were brought to pH 7 before injection. An hour later, blood was removed from the orbital sinus,(9) the animal was killed, and a sample of liver was frozen in a dry ice-acetone bath for later catalase assay. The blood was accurately measured, laked in cold water, diluted and assayed. Liver was weighed, thawed, homogenized in 1% nonionic detergent (Triton X-100),(10) diluted and assayed.

Three or 4 animals were used for each datum. For the most part, CF 1/Anl female mice, 6-8 weeks of age, were used; a few experiments were done with Charles River female mice of the same age, and no particular differences were noted between the 2 strains of mice in the parameters tested.

Results

Table 20 lists all compounds tested in either the in vivo or the in vitro system. The tremendous variation in dose injected in vivo is due to widely variable toxicity of the compounds. Generally, amounts injected were the maximum tolerable doses; occasionally, however, we were limited by the amount of agent available. Negative values indicate inhibition; positive values, at least in the in vitro system, probably indicate a stabilization of the dilute incubating enzyme mixture. Because only 3 or

*About 500 Beers and Sizer units.(8)

4 mice were used to obtain each in vivo datum, changes of less than 20-25% are considered probably of no significance in the in vivo tests.

In all cases in which liver was assayed, blood catalase was also measured. The blood results are not included in Table 20 because they were invariably negative.

TABLE 20
Effects on catalase in vivo of maximum tolerable dose of inhibitor, and effect in vitro of standardized doses

Compound	In vivo		In vitro		
	Dose, mmoles/kg	Change in liver catalase, %	Inhibitor concentration		
			2 x 10 ⁻² M	2 x 10 ⁻³ M	2 x 10 ⁻⁴ M
Change in catalase activity, %					
I. Substituted azoles					
A. Thiazoles					
2-Aminothiazole	2.5	-27°	-26°	+3	+6
2,4-Diaminothiazole	4.1	+11			
2-Aminobenzothiazole	0.2	-3	+1	±0	+10
2-Amino-2-mercaptiothiazole	0.5	+9	-32°	+8	+24
2-Mercaptothiazoline	4.2	-6	+13	+15	-2
3-Imino-5-thiono-1,2,4-dithiazolidine	2.2	-5	-65°	-10	-9
B. Oxazoles					
2-Mercapto-2-oxazoline			-19°	-1	±0
II. Diazoles					
A. Imidazoles					
Imidazole	7.4	+8	+21	+1	±0
2-Mercaptoimidazole	10.0	-66°	-76°	-2	+7
1-Methyl-2-mercaptoimidazole (Tapazole®) [Methimazole (USP)]	8.6	-7	+28	+21	+7
2-Mercapto-4(or 5)-methylimidazole	7.5	-3	+10	+9	+2
2-Mercaptoimidazoline	7.4	+17	+10	+9	+2
B. Pyrazoles					
4-Aminopyrazole	6.4	-63°	-100°	-30°	+4
4-Chloropyrazole	1.8	+20	-4	+5	+4
C. Indazoles					
5-Aminoindazole	1.9	-10	-24°	-3	-6
6-Aminoindazole	3.8	+4	-2	-8	+1
7-Aminoindazole	3.8	+7	-14°	-12	-1
D. Thiadiazoles					
2-Amino-1,3,4-thiadiazole	5.0	-21°	not done	+15	+13
2,5-Diamino-1,3,4-thiadiazole	8.6	-13			
5-Amino-2-mercapto-1,3,4-thiadiazole	3.8	-1	+22	+15	+7
2,5-Dimercapto-1,3,4-thiadiazole			-9	+15	+2
3-Hydroxy-4-methyl-1,2,5-thiadiazole			-36°	-38°	-28°
E. Oxadiazoles					
2-Amino-1,3,4-oxadiazole	11.9	+9	-9	-8	-2
2,2'-Bis(1,3,4,4H-oxadiazoline-5-thione)	3.7	+4			
2-Methyl-1,3,4,4H-oxadiazoline-5-thione	5.2	+1			
III. Triazoles					
A. No other ring substituents					
1,2,4-Triazole	7.2	-10	-90°	-22°	-4
3-Amino-1,2,4-triazole	26.8	-97°			
4-Amino-1,2,4-triazole	8.5	-13	-85°	-87°	-15°
5-Mercapto-1,2,4-triazole	5.0	-75°	-36°	+9	+12
4-Amino-3-mercapto-1,2,4-triazole	5.0	-12	+11	+13	+11
3-Amino-5-mercapto-1,2,4-triazole	4.3	-3	+22	+25	+30
3,4-Diamino-5-mercapto-1,2,4-triazole			-88°	-21°	-5
3-Furfurylidenamino-1,2,4-triazole	1.5	-39°			
3-Benzylidenamino-1,2,4-triazole	5.8	-22°			
3-Butylidenamino-1,2,4-triazole	7.2	-19°			
3-Amino-1,2,4-triazole glucose adduct	5.0	+12			
3-Amino-1,2,4-triazole xylose adduct	1.4	-13			
3-Amino-1,2,4-triazole mannose adduct	1.2	-13			

TABLE 20 (Contd.)

Compound	In vivo		In vitro		
	Dose, mmoles/kg	Change in liver catalase, %	Inhibitor concentration		
			2 x 10 ⁻² M	2 x 10 ⁻³ M	2 x 10 ⁻⁴ M
Change in catalase activity, %					
3-Amino-1,2,4-triazole ribose adduct	1.4	-13	+33	+21	+4
3-Acetylamino-1,2,4-triazole	10.0	+5			
3β-Aminoethyl-1,2,4-triazole	5.1	+2			
1-Methyl-5-mercaptop-1,2,4-triazole	1.3	-7			
3-Methyl-5-mercaptop-1,2,4-triazole	8.7	+4			
3-Phenyl-5-mercaptop-1,2,4-triazole	1.7	+3			
3-Mercapto-4-methyl-1,2,4,4H-triazole	4.3	+8			
1-Ethyl-1,2,4-triazole	10.3	+4			
1-Phenyl-1,2,4-triazole	0.9	+8			
4-(o-Tolyl)-1,2,4-triazole	0.3	+4			
3,5-Diketodihydro-1,2,4-triazole	1.0	-2			
3-Amino-5lor 6i-methyl-as-triazine	9.1	+11			
B. Thiazotriazoles					
5-Amino-thiazotriazole	1.3	-26°	-67°	-78°	-59°
IV. Tetrazoles					
5-Mercapto-1,2,3,4-tetrazole	5.0	-98°	-98°	-93°	-85°
5-Amino-1,2,3,4-tetrazole	11.8	-3	+18	+17	+6
1-Methyl-5-mercaptop-1,2,3,4-tetrazole	4.1	+1			
1-Isopropyl-5-mercaptop-1,2,3,4-tetrazole	6.9	+1	+24	+22	+6
1-tert-Butyl-5-mercaptop-1,2,3,4-tetrazole	3.2	+3	+21	+21	+17
1-Phenyl-5-mercaptop-1,2,3,4-tetrazole	2.8	+17	+14	+2	-4
Pentylene tetrazole	0.01	-5	+2	+2	+3
V. Other nitrogenous compounds					
Semicarbazide	0.4	-25°	-22°	-7	+4
Thiosemicarbazide	0.5	-1	-75°	-40°	-15°
S-methyl-isothiosemicarbazide	1.0	±0	-83°	-90°	-65°
Isonicotinic acid hydrazide	0.2	-22°	-5	+4	±0
1,1,3-Trimethyl thiourea	8.5	-4			
Aminoguanidine			-26°	±0	+3

For *in vivo* experiments, three or four mice were injected with a maximum tolerable dose of the agent in question, and the liver was removed for assay one hour later. For details of this and of *in vitro* techniques, see text. Negative figures represent inhibition; occasional positive figures (in the *in vitro* experiments) presumably indicate stabilization. Inhibitory effects considered significant, in both *in vivo* and *in vitro* experiments, are marked with an asterisk.

Table 21 summarized the *in vitro* testing for those compounds that proved active and presents results obtained with still greater dilutions of the more active agents.

TABLE 21

Summary of *in vitro* effectiveness of active inhibitors

Compound	Concentration for 50% inhibition, M	Effect on catalase activity, % Final inhibitor concentration			
		2 x 10 ⁻² M	2 x 10 ⁻³ M	2 x 10 ⁻⁴ M	2 x 10 ⁻⁵ M
5-Mercapto-1,2,3,4-tetrazole	6.0 x 10 ⁻⁵	-98	-94	-85	-18
S-methylisothiosemicarbazide	8.7 x 10 ⁻⁵	-86	-92	-72	-14
5-Mercapto-1,2,4-triazole	6.2 x 10 ⁻⁴	-85	-87	-15	-1
4-Aminopyrazole	3.8 x 10 ⁻³	-100	-30	+4	
Thiosemicarbazide	3.9 x 10 ⁻³	-75	-40	-15	
3-Amino-1,2,4-triazole	5.3 x 10 ⁻³	-90	-22	-4	
2-Mercaptoimidazole	9.0 x 10 ⁻³	-76	-2	+7	
3-Imino-5-thiono-1,2,4-dithiazolidine	1.1 x 10 ⁻²	-65	-10	-9	

Table 22 presents the results of assays of liver catalase one hour after the injection of standardized doses of the more active agents.

TABLE 22

Effect in in vivo on liver catalase of standardized doses of inhibitor

Compound	Concentration for 50% inhibition, mmoles/kg	Change in liver catalase at one hour, % Inhibitor dose, mmoles/kg		
		5.0	1.0	0.2
5-Mercapto-1,2,3,4-tetrazole	0.98	-98	-56	-8
5-Mercapto-1,2,4-triazole	1.76	-75	-33	-19
3-Amino-1,2,4-triazole	3.98	-79	-30	-10
2-Mercaptoimidazole	4.36	-54	-10	-7
S-methylisothiosemicarbazide		toxic	±0	not done

In Table 23, certain data of Tables 20 and 21 are pooled and rearranged to permit comparison of structure and activity.

TABLE 23

Correlation of structure with in vitro inhibition of catalase

	Amino	Mercapto
Non-cyclic	Aminoguanidine (-26, ±0, +3)	Thiosemicarbazide (-75, -40, -15)
Two ring N (diazoles)	4-Aminopyrazole (-100, -30, +4)	2-Mercaptoimidazole (-76, -2, +7)
Three ring N (triazoles)	3-Amino-1,2,4-triazole (-90, -22, -4)	5-Mercapto-1,2,4-triazole (-85, -87, -15)
Four ring N (tetrazoles)	5-Amino-1,2,3,4-tetrazole (+18, +17, +6)	5-Mercapto-1,2,3,4-tetrazole (-98, -94, -85)

Figures in parentheses are the in vitro catalase inhibitions in presence of 2×10^{-2} M, 2×10^{-3} M, and 2×10^{-4} M inhibitor. Minus signs represent inhibition; plus signs presumably indicate stabilization.

Discussion

The most effective catalase inhibitor we have yet encountered, no matter in what system tested, is 5-mercapto-1,2,3,4-tetrazole. In the in vitro system, it is more effective than AT by 2 orders of magnitude; in vivo, the ratio of effectiveness is about 4 to 1. Only slightly less

effective than the mercaptotetrazole in the in vitro system is S-methylisothiosemicarbazide. The in vitro activity of this compound was also observed earlier by Margoliash et al.⁽²⁾ However, this compound is totally ineffective in vivo when administered in a dose (1 mmoles/kg) which, though lethal, permits survival for one hour. Since at 1 mmole/kg the mercaptotetrazole is still a very effective inhibitor, it appears probable that S-methylisothiosemicarbazide is metabolized or somehow modified in vivo, or is unable to penetrate the hepatic cell.

It thus appears that in addition to the series of amino compounds discussed by Margoliash et al.,⁽²⁾ even stronger catalase inhibition is obtained from comparable mercapto compounds. These relationships are most clearly observable in Table 23. It is evident from this table that in the series of amino compounds, maximal activity is attained at the diazole stage, whereas in the case of mercapto compounds, continued introduction of nitrogen into the ring leads to continuing increase in inhibitory activity up to the tetrazole stage. Of particular interest is the fact that aminotetrazole has lost all inhibitory activity. Determinations of pK values* of the mercapto- and aminotriazole and -tetrazole provided no obvious explanation for this loss of activity.

Several compounds were available with both nitrogen and sulfur in the ring, and the results with these have been sufficiently interesting that we hope to continue investigations of this type of compound as more examples become available. We call particular attention in Table 20 to 5-aminothiatriazole and 3-hydroxy-4-methyl-1,2,5-thiadiazole.

The latter compound is of interest in two other connections also. First, it is the only instance known to us of an actively inhibitory compound of this general type having neither an amino nor a mercapto substituent. Second, it is one of very few active cyclic compounds containing more than a single substituent. Other instances are 3-imino-5-thiono-1,2,4-dithiazolidine, and the weakly active 4-amino-3-mercapto-1,2,4-triazole and 5-amino-2-mercaptotriazole.

A few words are in order concerning the more direct modifications of the AT molecule. These were of three types: a) the amino group was acetylated (3-acetylamino-1,2,4-triazole), b) the corresponding Schiff bases were prepared by reacting the free amino group with an aldehyde (e.g., 3-furfurylidenamino-1,2,4-triazole), and c) the sugar adduct was prepared. Of these three types, the first and last appear to be firmly bound, stable compounds, and little or no catalase inhibitory activity is evident from them. On the other hand, the Schiff bases act very much like the free AT, and inhibit catalase to about the same extent. Further, in aqueous solution, the ultraviolet absorption spectrum of furfurylidenamino

*We would like to thank Mr. Don Dennerlein for performing the titrations for pK determination.

triazole changes with time, while that of acetylaminotriazole does not. It is therefore concluded that the Schiff bases hydrolyze in aqueous solution, so that biological effects observed from these compounds are actually due to free AT.

Although blood catalase was also assayed each time liver catalase was assayed, no compound has yet been observed effectively and consistently to reduce the activity of the erythrocyte catalase in vivo. Two mechanisms have been postulated to account for the discrepancy between the effect of AT on the catalase of solid tissues and that of the erythrocyte: Feinstein and Dainko(11) suggested that AT could not penetrate the erythrocyte membrane, while Margoliash and Schejter(12) suggested that the erythrocytes are so rich in a dialysable, presumably sulphydryl, catalase donor that it is not possible for the AT to compete successfully for the catalase. Our present experiments tend to confirm the latter argument, since it seems very unlikely that of the many compounds herein tested and found active on liver catalase, none could penetrate the erythrocyte. Further, the only way in which we have yet been able to reduce erythrocyte catalase activity significantly is by the combined administration of mercaptotetrazole and glucose oxidase. The latter enzyme is considered to generate H_2O_2 in situ and thus tend to force the catalase in the direction of its catalatic reaction, rather than the peroxidatic, the mercaptotetrazole being better able to compete successfully with the former than the latter reaction. Whatever the mechanism, we have been able by this combination of reagents to reduce erythrocyte catalase significantly in vivo. Unfortunately, this treatment is on the borderline of lethality. Efforts are being made to adjust the treatment schedule so as to minimize toxicity while at the same time maintaining the inhibitory effect on erythrocyte catalase.

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EFFECT OF A SEROTONIN ANTAGONIST ON RADIATION LETHALITY

Robert N. Feinstein and John E. Seaholm

Recently Field, Mireles, and Dolendo⁽¹⁾ demonstrated that the serotonin antagonist (KB-95) [1-(N-methyl-piperidyl-4')-3-phenyl-4-benzyl-pyrazolone-5] significantly reduced mortality from HN₂ (nitrogen mustard). Because nitrogen mustard is in certain respects radiomimetic, the effects of KB-95 on the lethal effects of whole-body X-irradiation were tested.

Materials and Methods

Animals used were CF No. 1 female mice, 7-8 weeks of age, and Badger male rats, 6 weeks of age. The KB-95 was kindly provided by Dr. Leonard B. Achor of Sandoz Pharmaceuticals. The serotonin used was a product of the Upjohn Company. All injections were given intraperitoneally, in volumes of 0.01 ml/g of body weight. The KB-95 was administered as a suspension in 5% gum arabic, and the controls were given an equivalent volume of 5% gum arabic. X-irradiation was done with a GE Maximar machine; radiation factors were 250 kv, 15 ma, 0.5 mm Cu and 3 mm Bakelite filtration, HVL 1.72 mm Cu. Mice were irradiated at approximately 38 r/min; rats, at 114 r/min.

Results

Table 24 presents the pooled results of two experiments, indicating that prior injection of 400 mg KB-95 per kg significantly protects against 600 r of whole-body X-irradiation although the survival time of those mice that succumbed within 30 days increased by only 0.9 days. In single experiments (not listed) a) administration of the KB-95 2 hr after irradiation slightly increased survival (from 33 to 50%) and slightly decreased mean survival time (from 11.3 to 10.6 days), and b) administration of the KB-95 both before, and again 2 hr after, the irradiation was slightly less effective than preirradiation treatment alone (44% survival as compared to 56%). However, none of these differences is statistically significant.

The LD₅₀'s for whole-body X-irradiation, and the dose reduction factors (DRF) of KB-95, for both mice and rats (Table 25), indicate clearly that the agent, though protective, is only mildly effective.

Table 26 represents an attempt to determine whether or not the protective effect of KB-95 is due to its serotonin antagonism. The results again show protection by KB-95, even better protection by serotonin, and a bit less protection by the drug combination than by either drug alone.

TABLE 24

Effect of KB-95 on lethality of whole-body X-irradiation in mice

Treatment	No. of mice	Survival		Median survival time, days
		No.	%	
5% gum arabic, then 600 r	25	4	16	12.2
KB-95, 400 mg/kg, then 600 r	26	16	62	13.1
KB-95, 400 mg/kg, no X-ray	26	26	100	-

TABLE 25

Whole-body X-irradiation dose-reduction by KB-95

Species	LD ₅₀ (30), r.		Dose reduction factor
	Control	Treated	
Mice	615	687	1.12
Rats	650	710	1.09

A total of 72 rats and 170 mice was used for the LD₅₀ determinations.

TABLE 26

Effect of combined serotonin and KB-95 on lethality of whole-body X-irradiation in mice

Treatment	No. of mice	Survival		Mean survival time, days
		No.	%	
Saline, then X-ray	10	1	10	16.2
KB-95, then serotonin, then X-ray	10	7	70	13.7
KB-95, then X-ray	10	8	80	17.5
Serotonin, then X-ray	10	10	100	-
KB-95, then serotonin; no X-ray	5	5	100	-

Doses: X-rays, 700 r; KB-95, 500 mg/kg; serotonin, 100 mg/kg.

Discussion

The protective effect of the KB-95 against ionizing radiation differs from that against $\text{HN}_2(1)$ in at least one important respect, namely that its protective effects appear limited to preirradiation treatment, whereas KB-95 actually appears more protective against HN_2 if given after the HN_2 than if given before.

The experiment reported in Table 26 suggests that KB-95, an established antagonist of serotonin in the usual tests,* also somewhat antagonizes the radioprotective effect of serotonin. This is in interesting contrast to the experiments of van den Brenk and Elliott,(2) who found that several antagonists and antimetabolites of serotonin were not themselves at all radioprotective and, furthermore, completely negated the radioprotective ability of the serotonin when given together with that agent.

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*Unpublished data from laboratories of Sandoz, Inc.

PROGRESS REPORT: STUDIES ON OSSIFYING CARTILAGE

Klaus E. Kuettner* and Arthur Lindenbaum

A major purpose of this work is to study the physical and biochemical processes involved in the transformation of cartilage into bone. At present few of these changes are well understood. Since the collagen of ossifying cartilage appears to be no different from that of nonossifying cartilage, and no appreciable chemical or physical changes have been noted in collagen during ossification, our interest has been focused primarily on the mucopolysaccharide-containing portion. This contains functional groups, particularly SO_3H and COOH , that might be responsible for selective cationic binding. Since the cartilage mucopolysaccharides (mainly chondroitin sulfates A and C) occur mostly in the form of mucoproteins, part of our present work involves the extraction of these mucoproteins in a state of minimal degradation. As reported earlier^(1,2) simple water extraction of homogenized calf scapula cartilage has been found to provide relatively large quantities of mucoprotein in a nearly native state. Although few differences have been noted among mucoproteins extracted from different zones of calcifying cartilage by the chemical and physical-chemical analyses so far performed, there was a significant increase of bound calcium in the cartilage zone closest to the ossification "front."

In a continuing search for changes involved in the transformation of cartilage, we have begun to study the ion-binding properties of mucoprotein extracts by ion exchange and titration techniques. Earlier titration studies carried out on such polyelectrolytes by Wilander,⁽³⁾ using a nonautomatic procedure, yielded titration curves too crude for precise analysis of functional groups. More recently Helbert and Marini⁽⁴⁾ have also employed point by point titration for the estimation of sulfate, carboxyl and amino groups of heparin. For the present work an automatic titration technique (originally devised by Dr. H. Majer of CIBA, Basle, Switzerland) has been utilized for the titration of mucopolysaccharides and mucoproteins under nitrogen. Using this technique, titrations were performed with highly purified heparinic acid (Wilson and Company, 155 anticoagulant units/mg; converted to the H form with Dowex-50). As noted in Figure 39 the method of continuous titration yields a precise curve whose deflections allow not only the determination of the equivalent weight of this macromolecule (eq. wt. = 196.7) but also a calculation of the ratio (1: 2.63) between carboxyl and sulfate groups. We plan to utilize this method to determine similar ratios, total amounts of binding groups, and also pK values for mucoproteins and other macromolecules. It remains to be seen whether the protein moieties of these mucoproteins will undergo degradation in the presence of the titrating alkali solutions.

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Figure 39

Original titration chart obtained with 113.8 mg heparinic acid prepared from sodium heparinate (159 units/mg) and titrated with 0.0940 N NaOH. Superimposed are pH values in the horizontal axis and equivalent of base represented by vertical distances above the starting line. Tangential lines constructed to determine sulfate and carboxyl end-points (indicated by arrows: Point I, 4.21 equivalents; Point II, 5.81 equivalents) are also shown.

Another study has been undertaken* to devise a gentle separation and purification procedure for mucoproteins based on selective precipitation with dyes and other complex cationic substances capable of forming macromolecular complexes. Among these are 9-amino-acridine hydrochloride, "Rivanol" (6,9-diamino-2-ethoxyacridine lactate) and hexaminocobaltchloride. In this procedure the mucoprotein is completely precipitated with the dye, and the precipitate is then shaken with Dowex-50 in the potassium form to exchange the agent onto the resin, leaving the purified, presumably intact, potassium salt of the mucoprotein in solution. Possible alterations of purified mucoproteins obtained by this procedure may then be studied by the titration method described above. The chemical and physical analyses so far performed show no detectable changes in the purified material except for expected differences in ash content due to the change in cation. After precipitation of the mucoprotein the supernatant solution should be expected to contain all the other material extracted from the cartilage. Such solutions may eventually be worth examining for enzymatic activity or for other properties of metabolic interest.

Parallel immunological studies, including immunoelectrophoresis techniques, carried out in cooperation with Dr. G. Schumacher, The University of Chicago, have shown that mucoprotein extracts of calf scapula react with antibodies against serum albumin. We are now trying to determine the basis for this reaction, that is, whether the protein moiety of the mucoprotein is albumin (or at least immunologically related to albumin) or whether the precipitation of the mucoprotein with anti-albumin serum may be due to traces of contaminating serum components. Experiments in which rabbits were immunized with a mucoprotein extract over a period of about 10 weeks with relatively large amounts of antigen have so far been unsuccessful in producing precipitating antibodies either against the mucoprotein or against calf serum proteins.

*This work is being performed by Miss Florence Sheber, summer Resident Student Associate.

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PROGRESS REPORT: PLUTONIUM REMOVAL. VII. EFFECT OF DTPA
THERAPY AFTER 30 AND 80 DAYS ON THE REMOVAL OF
MONOMERIC PLUTONIUM FROM BONE AND LIVER

Joan F. Markley, Marcia W. Rosenthal, and Arthur Lindenbaum

Previous reports described the effect of therapy with diethylene-triaminepentaacetic acid (DTPA) begun at 1 hr or at 3 days on the distribution and excretion of monomeric plutonium in mice.^(1,2,3) It was established that the plutonium level in the skeleton reached a maximum 3-6 days after injection, after which it remained stable for at least 90 days. When therapy was initiated at 1 hr the skeletal burden was reduced to 23% of that in controls; about two-thirds of this effect was due to prevention of early deposition and the rest to removal of deposited plutonium.^(2,3) When the same course of treatment was begun 3 days after plutonium, the skeletal plutonium was lowered to about 50% of the controls; most of this effect was due to actual removal of deposited plutonium. The present experiment was conducted to test the effectiveness of DTPA therapy when plutonium deposition was maximal and sufficient time had elapsed for bone remodeling - 30 days. After this therapy the effect of an additional course of therapy at 80 days was investigated.

Materials and Methods

Fifty-four CF 1/Anl female mice, 72 days old, weighing 20-25 g, each received $3.0 \mu\text{c/kg}$ Pu^{239} intravenously, in 0.2 ml of a monomeric solution of plutonium citrate, pH 6.4, which was 95% ultrafilterable.⁽⁴⁾ They were then arbitrarily divided into 9 groups of 6 mice each. One group was killed at 3 days and another at 30 days after administration of plutonium. At 30 days, one group received saline injections intraperitoneally, 0.2 ml, once a day, for 18 days, and was then killed; the rest all received once daily intraperitoneal injections of 0.2 ml of CaNa_2DTPA , 500 mg/kg for varying time intervals up to 18 days. One DTPA-treated group was killed after 3, 9, 12 and 18 days of injections. The remaining two groups, after this 18-day DTPA therapy, were kept for an additional 33 days. They were then given a second course of 18 injections, either with DTPA or saline, and killed 98 days after plutonium administration.

A group of treated and control mice were kept in metabolism cages during treatment, 3 per cage, for collection of urine and feces. Samples of urine were collected every 3 days from the cages of the DTPA-treated mice and analyzed separately, while those from each cage of the control mice were pooled for the entire period. The feces from each cage were also pooled, except for the first 3-day collection from the DTPA-treated mice which was analyzed separately.

Livers and both femurs were removed and pooled from paired mice to make 3 samples for each group. Livers and feces were digested by a combination of muffling and wet-ashing techniques; all other samples were digested by wet-ashing alone. Plutonium content was assayed as previously described.(1) Skeletal plutonium content was estimated as mean femur content x 20.

Results

The distribution in bone and liver 3 days after plutonium injection was typical of that previously obtained with a monomeric solution; the content of plutonium in bone was $26.1 \pm 1.6\%$ and that in liver was $35.6 \pm 1.3\%$ of the injected dose (I.D.). Figure 40 shows the distribution of plutonium in bone and liver at 30 days and the effect of 18 days of DTPA therapy begun at this time on tissue distribution and excretion. As was expected from previous results with monomeric plutonium(1,2,3) the liver content was low (5% I.D.) and the bone content* relatively high (33% I.D.)

Figure 40. Effect of DTPA therapy on retention and excretion of plutonium in mice. All mice received plutonium intravenously. All bone and liver values are the means of determinations from 3 pairs of mice. Cumulative excretion values represent collections between 30 and 48 days only.

*Although the bone value in the controls showed a drop during the period of therapy, this is considered insignificant on the basis of the size of the standard errors and the previous results.(1,3)

at 30 days. Eighteen days of DTPA therapy removed essentially all of the remaining plutonium from the liver and nearly 50% of that deposited in the bone. The excreted plutonium was about equally divided between urine and feces.

No additional plutonium was removed from the bone of mice by the second 18-day course of DTPA therapy (begun at 80 days). At 98 days the bone value of these mice was 13.5% as compared with 13.3% I.D. in their controls. However, some increase (3% I.D.) in the excretion of plutonium was observed in these treated mice. A small portion of this amount evidently came from the liver which was reduced from 0.5% to 0.2% I.D.; the remainder must have been derived from other soft tissues.

Discussion

Comparison of these results with those obtained by DTPA therapy begun at 3 days^(1,3) shows that the plutonium in the bone of mice injected with the monomeric form is almost equally available to DTPA at 30 days and at 3 days. Smith⁽⁵⁾ observed removal of about the same proportion (one-half) of plutonium from the bone of rats treated at 38 days. It is also evident, from the negative results of the second course of DTPA therapy, that after plutonium was removed by DTPA given at 30 days, there was no more available in the bone for at least another 50 days.

The fact that the same amounts of skeletal plutonium were available at 30 and at 3 days may be explained in at least two ways: 1) Sufficient radiation damage occurred over 30 days to inhibit bone remodeling so that deposited plutonium remained exposed to the action of DTPA. Autoradiograms of dogs receiving $2.7 \mu\text{c/kg}$ or less of plutonium show inhibition of bone-remodeling as early as 9 days.⁽⁶⁾ If this were the case in these mice receiving $3.0 \mu\text{c/kg}$, additional therapy at 80 days would not be expected to have any further effect since the available plutonium would have been already removed. 2) Bone remodeling was not affected. Evidence that bone remodeling goes on after injection of plutonium is given by autoradiograms showing new bone formation in rats given $5 \mu\text{c/kg}$.⁽⁷⁾ Plutonium in trabecular and epiphyseal plate regions, which undergo relatively rapid remodeling, is made available by the remodeling processes up to 30 days. At 80 days, however, the remaining plutonium in these areas is quite diffuse and thus less available to DTPA. An alternate explanation for the failure of the second course of DTPA therapy is that cumulative radiation damage over the longer interval finally inhibited bone remodeling.

In view of the over-all slow rate of the remodeling process in the skeleton it seems unlikely that this process alone could account for the removal of 50% of the deposited plutonium by DTPA given at 30 days.

Clarification of the mechanisms responsible for this removal requires autoradiographic and histologic studies of mice similarly injected and treated, and, in addition, an assessment of the amount of skeletal plutonium removed by a single course of therapy at 80 days.

If the monomeric plutonium used in this experiment caused sufficient bone damage to prevent remodeling, its removal at 30 days may have no beneficial long-term effect. A current study⁽⁸⁾ of the effects of this level of monomeric plutonium on tumor incidence and survival in mice treated at 3 days also suggests that 30-day therapy would have little survival value. A better understanding of the relationships between physical state of the injected plutonium, therapeutic removal, and long-term survival is required, however, before final conclusions about the value of 30-day therapy after monomeric plutonium can be made.

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PROGRESS REPORT: PLUTONIUM REMOVAL. VIII. CHEMICAL REMOVAL OF MARROW AND ITS ASSOCIATED PLUTONIUM FROM BONE

Joan F. Markley, Marcia W. Rosenthal, and Arthur Lindenbaum

In a previous report⁽¹⁾ a chemical method for the separation of marrow plutonium from that on hard bone in the shafts of mouse and rat tibias, using the protein solvent Hyamine, was compared with a physical method. The two techniques gave the same results, indicating that Hyamine removed only the marrow with its associated plutonium without removing the plutonium on the surfaces of cortical bone. Using the entire tibia as a representative bone this method has now been used to determine the partition of both monomeric and polymeric plutonium between the marrow and the hard bone surfaces.

Adult female Sprague-Dawley rats and CF No. 1 mice were injected intravenously with 1.0 $\mu\text{C}/\text{kg}$ monomeric or polymeric plutonium. Rats were killed 3, 6 or 12 days after plutonium, mice at 3 days; all femurs and tibias were removed. The left tibias were wet-ashed, while the right tibias were crushed and extracted with Hyamine solution⁽¹⁾ before wet-ashing. Femurs were wet-ashed and the plutonium content of the whole skeleton was calculated as the mean femur content times 20.

The total skeletal plutonium and the proportional activity in marrow and hard bone of the tibias are summarized in Table 27. The difference in skeletal uptake of the two forms of plutonium is in accord with previous

TABLE 27

Plutonium content of marrow as determined by Hyamine separation

Species	Pu solution	Days after Pu	Pu, % of injected dose		Tibial Pu in marrow, * %
			Whole skeleton	Intact tibia	
Mouse	Polymeric	3	6.2 ± 0.2	0.19 ± 0.02	18.7 ± 4.9
Mouse	Monomeric	3	23.8 ± 1.6	0.83 ± 0.10	12.7 ± 3.2
Rat	Polymeric	3, 6, 12	11.7 ± 1.1	0.39 ± 0.02	25.7 ± 5.8
Rat	Monomeric	3, 6, 12	53.0 ± 9.4	2.00 ± 0.11	12.7 ± 2.9

*Percent tibial activity in marrow = $100 - \frac{\% \text{ injected dose in Hyamine-treated tibia}}{\% \text{ injected dose in intact tibia}}$

Values shown are the mean and standard error of the mean. Values for the mice are based on determinations from 3 pairs of animals. Values for the rats are the means of 9 determinations from single rats (3 for each time period).

results⁽²⁾ and indicates the degree to which the deposition of polymeric plutonium in the bone is minimized because of reticulo-endothelial uptake. The proportion of plutonium associated with the marrow is higher following administration of the polymeric solution.

These results on a single bone appear valid. However, the technique as applied to larger portions of the skeleton is as yet unsatisfactory,⁽³⁾ possibly because of loss of bone fragments in handling larger quantities of bone. Since it is important, from dosimetric and therapeutic considerations, to know the degree of partitioning of skeletal plutonium between marrow and hard bone, efforts to improve this chemical technique for marrow separation are being continued.

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PROGRESS REPORT: PLUTONIUM REMOVAL. IX. AN ULTRAFILTRATION TECHNIQUE FOR ESTIMATING THE COLLOIDAL PROPERTIES OF PLUTONIUM SOLUTIONS

Arthur Lindenbaum, William M. Westfall,
Marcia W. Rosenthal, and Joan F. Markley

Previous work⁽¹⁻³⁾ has demonstrated the relationship of physical state of plutonium injection solutions to variability in: 1) its distribution in the tissues of rats and mice, and 2) the extent of its subsequent removal by chelation therapy. A continuing problem encountered by many investigators has been the need for a precise knowledge of the colloidal properties of these solutions.

A crude but rapid and simple measure of colloidal state developed in this laboratory is the determination of the fraction of plutonium ultrafilterable through a cellophane membrane after centrifugation.⁽⁴⁾ This procedure, based on the technique originated by De Waard,⁽⁵⁾ is as follows: Cellophane bags, tied at one end, are prepared from $\frac{3}{8}$ in. Visking tubing. Plasticizers are removed from the bags by boiling with four successive changes of double-distilled water for 15 minutes each. Before use the bags are inflated by compressed air and dried under an infrared lamp. One-ml aliquots of plutonium solution to be ultrafiltered are transferred to duplicate bags by means of long-tipped pipettes. Each bag is suspended about 1.5 cm above the bottom of a plastic (cellulose acetate or polycarbonate) tube by folding the mouth of the bag over the lip of the tube and placing a rubber stopper in the tube. The assembly is centrifuged at 1,000 x g for 20-45 min. About 0.05 to 0.2 ml of ultrafiltrate, sufficient for duplicate radioactivity analyses, may be collected from each 1.0 ml sample. Duplicate aliquots of ultrafiltrates and solutions before ultrafiltration are spread on stainless steel planchets, dried, heated to incandescence and the Pu²³⁹ activity determined by alpha-counting in a windowless, proportional counter. The fraction of plutonium ultrafiltered is determined using the following equation:

$$\% \text{ ultrafilterability} = \frac{\text{cpm}/v \text{ (ultrafiltrate)}}{\text{cpm}/v \text{ (unfiltered soln.)}} \times 100$$

where v represents any convenient known volume. Sample counts obtained generally agree within 2-3% of one another. The procedure may be completed within 1 hr after preparation of the injection solution.

The pore size of the cellophane membranes used for this work was estimated by ultrafiltering a solution of highly purified cytochrome c under the same conditions. No detectable cytochrome c was found in the ultrafiltrate, indicating that the pore size does not allow passage of particles having a size larger than that of a globular protein of molecular weight 11,700.

Cellulose membranes, even from the same manufacturer, differ greatly in pore size,⁽⁶⁾ so that for the most precise work the pore size should be determined for each batch of cellulose tubing. It should be noted that the ultrafiltration procedure offers no information on the distribution of charge or particle size; the material in the ultrafiltrate is not necessarily monomeric but may be present as relatively short-chain polymers.

Table 28 is a compilation of randomly selected data brought together to illustrate the relationship between ultrafilterability of plutonium injection solutions and the subsequent distribution of plutonium in mouse tissues. In general, with increasing colloidal properties (decreasing ultrafilterability) there is less plutonium deposited in the bone and more in liver and spleen.

TABLE 28

Distribution of plutonium in mice 3 days
after intravenous injection

% ultrafilterable Pu in injection solution	% injected Pu		
	Bone	Liver	Spleen
93	32	34	-
83	21	37	<0.2
64	14	39	1.0
39	33	41	0.4
35	34	48	0.4
16	24	44	0.5
6	7	90	2.5

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PROGRESS REPORT: PLUTONIUM REMOVAL. TUMOR INCIDENCE STUDIES. V.

Marcia W. Rosenthal, Joan F. Markley,
and Arthur Lindenbaum

The preceding report in this series⁽¹⁾ described a long-term experiment in which mice injected intravenously with $2.7 \mu\text{c/kg}$ of a monomeric form of Pu^{239} were treated with single daily intraperitoneal injections of 500 mg/kg of diethylenetriaminepentaacetic acid (DTPA) for 12 days, beginning either 1 hr or 3 days after plutonium administration. Survival and bone tumor incidence data at 400 days were summarized and compared with those following polymeric plutonium. This report summarizes the significant developments between 400 and 650 days following monomeric plutonium.

The last mouse in the Pu control group (which received only saline after Pu) died at 501 days. About four-fifths of these mice had at least one malignant bone tumor at death (Table 29). DTPA therapy, either at 1 hr or 3 days, significantly prolonged survival but not to the level of controls receiving only DTPA.

TABLE 29

The influence of DTPA therapy on tumor incidence and survival up to 650 days after monomeric plutonium

Group	Per cent survival		Tentative tumor incidence*		
	501 days	650 days	501 days		650 days
			Proportion	%	
Pu + saline	0	-	41/51	80	-
Pu + DTPA at 3 days	31	6	26/35	74	36/48
Pu + DTPA at 1 hr	72	32	6/15	40	22/36
DTPA only	84	52	0/16	0	1/48
					2

*Tumor incidence = proportion of mice dying after the latent period (153 days) that had at least one malignant bone tumor at death. Bone tumors were diagnosed only on the basis of roentgenographic examination ("positive" plus "probable" tumors) as described in Reference 1.

In the group of Pu-injected mice treated at 1 hr with DTPA no mouse had a malignant bone tumor at death until 354 days, about twice as long an interval as the 153-day latent period of the Pu controls. After 408 days, when the next mouse with a tumor died, the rate of appearance of tumors increased, so that by 650 days the tumor incidence was about 60% (Table 29). Thus, the reduction of the skeletal plutonium level in this group to one-quarter of the level observed in control mice⁽¹⁾ results in a considerable prolongation of the latent period, but not in a corresponding reduction in bone tumor incidence.

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LYSOSOME PARTICLES AND SUBCELLULAR DISTRIBUTION OF POLYMERIC TETRAVALENT Pu^{239}

Yueh-Erh Rahman and Arthur Lindenbaum
(with the technical assistance of William M. Westfall)

Summary

Tissue fractionation studies were made of livers of rats, 1 day, 8 days, and 28 days after a single intravenous injection of $0.5 \mu\text{c}$ of polymeric plutonium [Pu^{239} (IV)]. The subcellular distribution of the plutonium was found to be nearly identical with the distribution of the acid phosphatase, indicating concentration of Pu in the lysosomal fraction.

Based on the release of acid phosphatase from liver lysosomes, no difference in sensitivity to a non-ionic detergent, Igopal-630, was found between the lysosomes of plutonium-injected rats and of uninjected controls for 14 days after plutonium injection.

COMPLEX PATTERNS IN THE MITOCHONDRIA OF A RADIOSENSITIVE GIANT AMOEBA

Edward W. Daniels and Evelyn P. Breyer

During interphase the mitochondria of the radiosensitive amoeba, Pelomyxa illinoensis, have relatively few tubules. Most of these are around the periphery; the central area is relatively large and clear. Other work suggests that this mitochondrial form is related to low cytochrome oxidase content, and we recently found that this amoeba has a very low quantity of cytochrome oxidase. In structure the mitochondrion can be viewed as a vesicle with two membranous walls that are separated by a space of about 140 Å. Invaginations of the inner membrane into the vesicle form tubules, which often become branched. The lumina of the tubules are continuous with the 140 Å space, and this system often contains electron-dense material. During mitosis complex patterns are observed in the mitochondria caused by the alignment of tubules which appear either as parallel wavy lines when the mitochondrion is cut lengthwise, or as rows of open tubules when it is cut in cross section. These patterns are often found only at one end and occupy but a fraction (ca. 1/4) of the interior space of the mitochondrial vesicle; tubules are often absent from the remainder of the mitochondrion, which resembles an empty vesicle. Transition forms showing intermediate stages between these and the type of mitochondria found in interphase can be seen within a given mitotic cell, but only during mitosis. These include "ring" forms, with the tubules pressed against the periphery due to an apparent swelling of the interior, and forms with little or no tubular structure. During mitosis some of the mitochondria appear to lose electron-dense material from their tubular systems into the adjacent cytoplasm. Everted extensions of the outer mitochondrial membrane wall have been seen on rare occasions, and these contain electron-dense material. Complex patterns in mitochondria are found most frequently in prophase and metaphase, not in mature interphase.

ISOLATION OF CELL COMPONENTS FROM AMOEBA PROTEUS

C. Ward Kischer,* Edward W. Daniels, and Anna Kane Laird

Amoebae that have received supralethal doses of ionizing or ultraviolet radiation live normally and reproduce indefinitely if irradiation is followed by fusion with unirradiated amoebae of the same species^(1,2) or by the microinjection of cytoplasm from such normal amoebae.^(3,4) Because of the permanence of the radiorestorative effect of normal cytoplasm, it seems likely that the effective elements include nucleoprotein moieties, capable of self-reproduction.

The lethal effects of ionizing and ultraviolet radiation in mammalian organisms⁽⁵⁾ and in mammalian cells in tissue culture⁽⁶⁾ respectively, have been prevented by the postirradiation administration of highly polymerized DNA obtained from normal, unirradiated cells and tissues.

These results together suggest that a protoplasmic fraction of amoebae, especially one containing nucleoprotein elements, might prove to be the radiorestorative agent. The present work was directed toward isolating and identifying the protoplasmic constituents of amoebae, in the hope that eventually one might be able to produce radiorestoration by injection of such chemically and morphologically defined material.

In previous experiments⁽⁷⁾ we treated supralethally irradiated amoebae (Pelomyxa illinoensis) with highly polymerized DNA and RNA extracted by chemical procedures from unirradiated amoebae of the same species; however, no increase in survival was observed.

Materials and Methods

Cell fractionation of amoebae. Amoeba proteus cells were homogenized, and cell components were isolated by an arbitrary procedure of centrifugal fractionation, based on previous experience with mammalian tissues.

The homogenizing medium was 8.5% sucrose, containing CaCl_2 , 0.00018 M, a standard medium for work with mammalian tissues. The homogenate was centrifuged in conical tubes in the International centrifuge at approximately 2000 rpm; a double sediment was obtained, green on the bottom and white above. A crude separation of these two fractions was accomplished by gentle pipetting. The supernatant fluid was centrifuged at 19,000 x g, and the sediment was saved for microscopic examination; the supernatant was centrifuged at 48,000 x g; this sediment was also saved, and this supernatant was centrifuged at 105,000 x g.

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The fractions were studied by both light and electron microscopy. For electron microscopy, suspensions of the isolated particles were diluted and prepared by the critical point drying method of Anderson,(8) which avoids flattening the material against the grid; such preparations were then shadowed with platinum. Pictures of only two fractions, the 19,000 x g and the 105,000 x g sediments, are illustrated in this report.

Light Microscopy

The green sediment contained nuclei, skins (plasmalemma) of amoebae, and many food vacuoles, but cytoplasmic granules were relatively rare. The white sediment, in addition to the above, contained cytoplasmic granules of many sizes. The 19,000 x g sediment contained cytoplasmic particles which were small, dense and optically black; they were mostly round or irregular, with few rod-forms; the size range extended to the limits of visibility. The 48,000 x g sediment consisted of a moderately heavy suspension of small dark bodies, grayish and poorly defined and irregular in shape. The 105,000 x g sediment was not examined under the light microscope.

Electron Microscopy

Electron micrographs of the particles characteristic of the 19,000 x g sediment are shown in Figure 41. They are usually round or oval and have a complex inner structure that seems to be viewed through an outer transparent membrane. The membrane is often folded, as though it had been stretched during preparation and then had collapsed upon the more rigid inner core. In size and shape these particles are similar to sectioned mitochondria of whole amoebae (Figure 42). The mitochondria of these amoebae are known from previous study to have a tubular inner structure rather than the cristae characteristic of most mammalian tissues.(9) We tentatively suggest that the particles seen in the whole mounts of Figure 41 are mitochondria, and that the complex inner core visible through the outer membrane consists of the tubules with which we are familiar in sectioned material.

The networks of threads characteristic of the 105,000 x g sediment are shown in Figure 43. The linear elements of the network are multi-stranded, the unit fibrils separating at nodes and continuing uninterrupted along the branches of the network. The unit threads appear to be coiled or beaded, and are less than 10 m μ wide. The fibrils appear to be surrounded by a transparent sheath; we believe this sheath to be an artifact, however, because it is not shadowed and, therefore, could not have been present at the time of shadowing. Such sheaths have been demonstrated in similar preparations of DNA threads,(11) and apparently are produced by a condensation of carbon on such three-dimensional structures in the electron beam of the microscope.(11)

Figure 41. Particles characteristic of the 19,000 x g sediment from A. proteus. They are about 0.1 to 0.6 μ in diameter; the units of the inner core are about 20-30 m μ in diameter. In the background are many smaller particles, about 20-60 m μ in diameter.

Figure 42. Electron micrograph of a section through A. proteus, after centrifugation of the intact cell at 700 x g for 30 min. The mitochondria measure about 1 to 1.5 μ in diameter; the tubules within them are about 4.5 m μ in diameter.

Figure 43. 105,000 $\times$ g sediment from A. proteus. The elements of the reticulum consist of optically dense, multistranded fibers; individual strands separate and continue uninterrupted at nodes of the reticulum (arrows). These unit strands are less than 10 $m\mu$ in diameter, and appear to be coiled or beaded. The outer transparent sheath is probably an artifact, because it is not shadowed, and was therefore not present during the shadowing procedure that preceded examination under the electron beam. Similar sheath-like artifacts have been observed surrounding DNA fibrils,⁽¹¹⁾ and are believed to be due to condensation of carbon on a relatively cold specimen in the electron beam.⁽¹¹⁾ The round particles dispersed throughout the field are presumably the same elements as seen contaminating the 19,000 $\times$ g fraction; they are about 20 to 30 $m\mu$ in diameter.

Elements of this fraction have not yet been identified in sections of whole amoebae. They might be cytoplasmic elements not previously identified, or, since the threads resemble DNA fibrils, they might originate in nuclei broken during homogenization. Biochemical studies of the fractions will be necessary to establish the nature of the morphological components.

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PROGRESS REPORT: SOLUBLE PROTEINS FROM CELL NUCLEI

A. D. Barton

Various lines of evidence suggest that the nucleus is not only a repository for genetic information, but also imposes a continuous influence on the activities of the cell. This influence may be exerted in part by a more or less continuous dissemination of genetically determined information to the cytoplasm. The ultimate aim of the present study is to obtain more information concerning this role of the nucleus in cell metabolism.

It has been shown that cell nuclei lose substantial amounts of protein when isolated in aqueous media. Nevertheless, we found in early studies that liver cell nuclei isolated in sucrose media still retain soluble proteins that can be extracted with ionic media, i.e., proteins retained in the nucleus by ion binding. It appeared that these proteins might be important in nuclear function since relatively small changes in ion concentration may determine whether they are bound to or liberated from the nucleoprotein structures in the nucleus. Accordingly, these proteins are being isolated from rat liver nuclei and studied in moving boundary electrophoresis.

In a recent progress report⁽¹⁾ we described experiments dealing with the isolation of the nuclei and the extraction of these nuclear proteins, and we indicated that the electrophoretic pattern was responsive to changes in the physiological condition of the animal. The selective effects obtained by using various combinations of phosphate and calcium chloride in the isolation procedure indicate that these proteins are not retained by random ion binding, in which the most basic proteins would be expected to be bound the most firmly. On the contrary, the results indicate that the different protein classes are bound at specific sites, and that each binding process has individual characteristics, the presence of divalent metal ions being important in some cases.

The present report deals with more extensive experiments concerning the response of the soluble nuclear proteins to changes in the physiological state of the animal. In these experiments we have studied the response to prolonged starvation and recovery, to prolonged administration of a liver carcinogen, and to administration of hydrocortisone.

In the starvation experiments, the animals were deprived of food but allowed access to water ad libitum; after 5 days of this starvation their soluble nuclear proteins yielded patterns like those shown in Figure 44A. The corresponding pattern for animals that were similarly starved and then allowed access to food for one day are shown in Figure 44B. The patterns from the starved animals appear relatively normal although, as in all

deprivation patterns, there is even less indication of individual peaks in the mobility range between +1 and -1 than there is in preparations from normal animals. After one day of refeeding individual components are well displayed, the most prominent change being the relatively large increase in the component at -0.6 in the descending pattern.

In the experiments with the liver carcinogen, animals were maintained on a semisynthetic diet [diet #2 of Miller et al.(2)], to which was added 0.06% 4-dimethylaminoazobenzene. After five weeks, animals still in the first phase of the precancerous period⁽³⁾ were selected on the basis of low body weight and low liver weight. After six weeks on the diet, animals in the second precancerous phase, after extensive cell division had begun, were selected on the basis of increased body weight and liver weight, and these selections were confirmed by subsequent examination of histological sections. The patterns from the animals in the first phase (Figure 45A) resemble those from normal or starved animals. The striking change in the patterns from the animals in the second phase (Figure 45B), when the animals were adapting to the presence of the carcinogen in the diet, is the increase in the component at -0.6 in the descending pattern.

In all of the experiments described, characteristic peaks appear in the electrophoretic patterns whenever the animals pass from a quiescent state to one in which there is active synthesis of liver constituents. The most probable function for the nucleus in this "induced" synthesis of new liver constituents is the synthesis of messenger RNA, and a more direct test of this possibility seemed desirable. It is well known that administration of hydrocortisone induces in the liver the synthesis of a number of enzymes that degrade amino acids; and recently it has been demonstrated^(4,5) that the hormone actually induces the synthesis of the specific messenger RNA's which then function in the synthesis of the enzymes.

Accordingly, a suspension of hydrocortisone in saline was administered intraperitoneally to adult rats (50 mg/kg), and the animals were killed 1 to 3 hr later. In each case, the electrophoretic patterns of the soluble nuclear proteins yielded characteristic peaks, a typical example being shown in Figure 46. Here again, the component at -0.6 is prominent. Thus, administration of a hormone that is known to induce synthesis of messenger RNA in the liver produces the characteristic peaks in the electrophoretic patterns of the soluble nuclear proteins.

Monod et al.⁽⁶⁾ have shown that the activity of certain enzymes can be controlled by allosteric attachment of activator and substrate molecules at different sites on the enzyme molecule. In view of the unique control that such a system provides, they have postulated the existence of protein repressor molecules, whose presence at specific loci on the DNA prevents the synthesis of messenger RNA at those loci unless the appropriate inducer is attached to the repressor protein. If the allosteric principle is

Figure 44. Moving boundary electrophoresis of the soluble nuclear proteins; buffer⁽⁷⁾ sodium veronal 0.02 M, sodium chloride 0.03 M, pH 8.6; protein concentration 0.6 to 1%; voltage gradient 4.4 to 4.6 volts/cm; time 180 min. The mobility scale in $(\text{cm}^2 \times 10^5)(\text{v}^{-1})(\text{sec}^{-1})$ is given at the bottom, and, in each case, the ascending pattern is on the left and the descending pattern on the right. A. Soluble nuclear proteins from animals starved for five days. B. Comparable preparation from similarly deprived animals after one day of refeeding. Note the appearance of discrete components, particularly the one at -0.6 in the descending pattern.

Figure 45. Soluble proteins from animals on a semisynthetic diet containing 4-dimethylaminoazobenzene; details are given in the text. A. After five weeks on the diet; animals in the first phase of the precancerous period. B. After six weeks on the diet; animals in the second phase of the precancerous period, becoming adapted to the presence of the carcinogen in the diet. Note the prominence of the -0.6 component in the descending pattern. The mobility scale in $(\text{cm}^2 \times 10^5)(\text{v}^{-1})(\text{sec}^{-1})$ is given at the bottom.

Figure 46. Soluble nuclear proteins from animals given hydrocortisone acetate (50 mg/kg) three hr previously. Note the appearance of individual components, including the one at -0.6 in the descending pattern. The scale at the bottom is $(\text{cm}^2 \times 10^5)(\text{v}^{-1})(\text{sec}^{-1})$.

applied to these hypothetical repressor proteins it would provide for continuously variable control of cell metabolism by simultaneous summation of the effects of inducers, repressors, and hormones on the synthesis of messenger RNA.

The soluble nuclear proteins that we are studying are attached by ion binding to specific sites in the nucleus, and each binding process has individual characteristics. In situations in which messenger RNA is being synthesized, these proteins show characteristic electrophoretic peaks. All of our results are compatible with the hypothesis that some of our nuclear proteins function as adaptor molecules to which inducers can be attached, and that the resulting combination is present on the DNA when the synthesis of messenger RNA is taking place.

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BIOSYNTHESIS OF METHIONINE IN SACCHAROMYCES CEREVISIAE; PARTIAL PURIFICATION AND PROPERTIES OF S-ADENOSYL- METHIONINE:HOMOCYSTEINE METHYLTRANSFERASE

Stanley K. Shapiro, David A. Yphantis, and Aldona Almenas

The biosynthesis of methionine from homocysteine may occur via transmethylation or via one-carbon addition from formate or serine. The transmethylation of homocysteine with S-adenosylmethionine as the methyl donor has been described in microorganisms, plant tissues, and rat liver.⁽¹⁾ The utilization of acetoethetin, dimethyl- β -propiethetin, and betaine as methyl donors for the methylation of homocysteine in mammalian liver has been studied extensively,⁽²⁾ but these compounds are inactive in microbial systems. Another mechanism of methionine biosynthesis from homocysteine involves the formation of the methyl group from the β -carbon of serine via tetrahydrofolate (THF). The latter mechanism has been investigated in *E. coli*.^(3,4) Recently it has been shown that S-adenosylmethionine, previously established as a methyl donor in the biosynthesis of methionine, is also involved in the biosynthesis of methionine via the THF-serine mechanism.⁽⁴⁾ In *S. cerevisiae*, however, S-adenosylmethionine:homocysteine methyltransferase is the only mechanism that has been demonstrated.⁽¹⁾ In order to obtain insight into the mechanism and significance of the latter reaction, the purification of the enzyme was undertaken. The purpose of this report is to describe the purification procedure and the general properties of the partially purified enzyme.

Experimental Procedure

Compounds. S-Methylmethionine was synthesized according to the method of Toennies and Kolb,⁽⁵⁾ and S-adenosylmethionine was isolated from yeast cells by the procedures of Schlenk et al.⁽⁶⁾ Commercial L-methionine- $C^{14}H_3$ or $-S^{35}$ and DL-methionine- $2C^{14}$ were used to prepare the correspondingly labeled sulfonium compounds. All other compounds were commercial products.

Column Chromatography. DEAE-cellulose was obtained from the Brown Chemical Co., Berlin, N.H. A column 6 cm in diameter was filled to a height of 20 cm with the cellulose over a 2-3 mm layer of sand. The cellulose was added as a slurry in 0.5 M phosphate buffer, pH 6.8, and washed with 700 ml of this buffer at a drop rate of 2 ml per minute. The column was equilibrated with 10 liters of 0.01 M phosphate buffer, pH 6.8, at a flow rate of 10 ml per minute. For use in the batch treatment, DEAE-cellulose was prepared in the same way, removed from the column, dried in an oven at 60° for two days, and used as a powder. Hydroxylapatite was prepared according to the method of Tiselius et al.⁽⁷⁾ and packed in a 1 x 20 cm column over 2-3 mm of sand. Before use, the column was

equilibrated with 2 liters of 0.01 M phosphate buffer, pH 7.2. Sephadex G-25 (coarse), Sephadex G-75 (medium), and DEAE-Sephadex A-50 were obtained from the Pharmacia Corp., Rochester, Minn. Sephadex G-25 was prepared in distilled water and after removal of fine particles was packed in a 2 x 30 cm column according to the directions of Flodin.⁽⁸⁾ Sephadex G-75 was washed with 50% glacial acetic acid, washed with water and after removal of fine particles as above was suspended in 0.2 M phosphate buffer, pH 6.8. Before use the suspension was deaerated with a water aspirator. The final product was packed in a 0.9 x 200 cm column with 0.2 M phosphate buffer and equilibrated with 500 ml of the same buffer. DEAE-Sephadex was prepared according to the manufacturer's instructions, packed in a 0.5 x 13 cm column, and equilibrated with 100 ml of buffer consisting of 0.02 M phosphate - 0.05 M NaCl, pH 7.0.

Paper Chromatography. Ascending or descending chromatograms were prepared with Whatman No. 1 paper, and the following solvents were used: 1) 1-butanol-acetic acid-water (60:15:25 v/v); 2) 1-butanol-ethyl ether-water (78:18:6 v/v); 3) 1-butanol-formic acid-water (77:10:13 v/v); 4) pyridine-water (65:35 v/v). Amino acids were detected with a spray of 0.25% ninhydrin in acetone containing 1% acetic acid, and sulfur-containing compounds were located with a platinic iodide spray.⁽⁹⁾ Radioactivity was detected on the chromatograms with an automatic scanning and recording device.⁽¹⁰⁾

Enzyme Assay. The enzymatic synthesis of methionine was measured by the tracer assay of Shapiro and Yphantis.⁽¹⁾ Standard assays were performed with 0.1 ml of an appropriate dilution of enzyme, 4 μ moles of L-homocysteine, 4 μ moles of S-methyl-L-methionine- $C^{14}H_3$, or S-adenosyl-L-methionine- $C^{14}H_3$, 0.05 μ moles of Zn^{++} in 0.1 M phosphate buffer, pH 7.0, in a total volume of 0.5 ml. After incubation for 1 hour at 37°, the reaction was stopped by rapid cooling to 0° and 0.2 ml of the reaction mixture was placed on a short ion-exchange column (0.5 x 2 cm of Dowex 50 x 4 in Li^+ form). The radioactive substrates were retained by the columns, whereas the product of the reaction, methionine, was collected quantitatively by washing with three aliquots of 0.25 ml distilled water. Since methionine was the only radioactive product formed in the reactions with yeast extracts, the amount of radioactivity in the effluent was directly proportional to enzyme activity.⁽¹⁾ In all cases the results were calculated after subtraction of the control values obtained without L-homocysteine or without enzyme. In standard assays S-adenosylmethionine- $C^{14}H_3$ was used at a specific activity of 20,000 cpm/ μ mole, and the resultant methionine was calculated on the basis of the same specific activity as the methyl donor. S-Methylmethionine- $C^{14}H_3$ was used at a specific activity of 40,000 cpm/ μ mole and methionine was determined on the basis of a specific activity of 20,000 cpm/ μ mole.

One unit of activity is defined as the amount of enzyme that catalyzes the synthesis of 1.0 μ mole of methionine per hour under standard assay

conditions. Protein concentrations were determined by the method of Lowry et al.⁽¹¹⁾ or by optical density measurements at 280 m μ in a Beckman DU spectrophotometer. One optical density unit was considered equivalent to one mg protein.

Analysis of Reaction Products. Methionine was isolated from reaction mixtures as methionine sulfoxide, following the methods described previously for protein hydrolysates.⁽¹²⁾ The ninhydrin method of Yemm and Cocking was used to determine the concentration of the amino acid after pretreatment with Dowex 50 according to the procedure of Kornberg and Patey. The other reaction product, S-adenosylhomocysteine, was isolated by elution from paper chromatograms developed in solvent #1. Its concentration was determined with a Beckman DU spectrophotometer and confirmed with the orcinol test.⁽¹³⁾ Appropriate blanks from eluates of the same chromatograms were used in all cases. The isolated reaction products were checked for purity in the solvent systems described above.

Radioactivity. Assay of radioactivity was carried out in a Packard Tri-Carb Spectrometer. All samples were made up in a total volume of 1.0 ml, and 20 ml of scintillation fluid were added to each vial. The fluid consisted of 0.4% 2,5-diphenyloxazole in equal parts of toluene and absolute alcohol.

Preparation of Cell-free Extracts. Commercial baker's yeast yielded enzyme preparations of activity comparable with those of pure cultures and was used as the starting material in all subsequent work. Five hundred grams of cells and 32.6 g of NaHCO₃, suspended in 250 ml distilled water, were heated to 37° in a water bath, and 88 ml of toluene were added. The suspension was incubated for 1.5 hr, diluted with an equal volume of ice water, and rapidly cooled to 0°. After centrifugation at 5000 x g for 30 min, the supernatant fluid was filtered through four layers of cheesecloth. The filtrate was centrifuged at 15,000 x g for 30 min and the supernatant fluid served as a crude toluene enzyme extract.

Results

Alcohol Fractionation. One per cent DL-methionine was added to the toluene extract. After one hour at 1° most of the amino acid was in solution. Absolute alcohol at -20° was added to 100 ml of this mixture at a rate of 2 ml per minute, and for larger batches the rate of addition was scaled upward proportionately. In all cases the toluene extract was mixed vigorously and maintained at a temperature below 3°. As soon as the alcohol concentration was high enough to prevent freezing, the mixture was cooled to -10°. Sufficient alcohol was added to attain a concentration of 53%. After standing for 16 hr at -15° the mixture was centrifuged at 15,000 x g for 30 min. Absolute alcohol at -20° was added to the supernatant fluid to attain a concentration of 70%, while temperature was

maintained at -15° . After one hour at this temperature the mixture was centrifuged as before. The precipitate was dissolved in 0.01 M phosphate buffer, pH 6.8 (25 ml buffer per liter of toluene extract) and dialyzed for 16 hr in the same buffer.

As may be seen in Table 30, the alcohol fractionation in a typical experiment resulted in a 23-fold purification with 25% recovery of the original activity. Furthermore, the resultant 280/260 $m\mu$ ratio changed from 0.60 for the toluene extract to an average of 1.2 for the 53-70% alcohol fraction. The loss of most of the nucleic acid made it possible to rely on the optical density at 280 $m\mu$ to measure protein concentration in all subsequent steps. Occasional cross checks with the method of Lowry et al. (11) showed good agreement in the estimation of protein concentration. In most cases the 53-70% alcohol fraction was quite stable and has been stored at -15° for 2-3 years with virtually no loss in activity.

TABLE 30

Purification of methyltransferase of S. cerevisiae

Treatment	Total protein, mg	Total activity, units	Yield, %	Specific activity, units/mg protein	Purification factor	Ratio of activities, AM/MM*
Toluene extraction	60,000	10,000	100	0.17	-	0.52
Alcohol fractionation	650	2,550	25.5	3.9	23	0.51
DEAE-cellulose chromatography	48	735	7.4	15.4	91	0.54
Hydroxylapatite chromatography	0.54**	49**	0.5**	89.8	530	0.55

*AM = S-adenosylmethionine; MM = S-methylmethionine.

**These values were obtained with $1/3$ of the DEAE-cellulose eluates.

DEAE-Cellulose Chromatography. DEAE-cellulose provided a good method of purification (Table 30). The method is a compromise between good yield and high purification. A typical experiment with a large batch of enzyme is shown in Figure 47. Twenty-five ml of enzyme (53-70% alcohol fraction) containing 650 mg protein and 2550 units of activity were added to the 6 x 20 cm DEAE-cellulose column at a rate of 1 ml per minute. Elution was begun with 750 ml of 0.02 M phosphate buffer, pH 6.8, at a rate of 4 ml per minute. A fraction collector was used to collect samples with a volume of 8.8 ml per tube. As may be seen in Figure 47, the inactive protein is eluted in a discrete peak immediately after the hold-up volume of 180 ml has passed through the column. Beginning with tube 86, a gradient elution was begun with the addition of 0.2 M phosphate buffer to 200 ml of 0.02 M phosphate buffer in a constant volume mixing

chamber. The enzyme is eluted in a rather sharp peak at an estimated phosphate concentration of 0.09-0.12 M (Figure 47). The peak tubes contained 30% of the enzyme added to the column, and the purification factor was 4 (Table 30). The total time of exposure of the enzyme to the cellulose was just 5 hr. Slower elution did not improve significantly the resolution of the enzyme from the total protein during the gradient elution, and the longer exposure to cellulose resulted in greater losses of total activity. Likewise, increasing or decreasing the slope of the gradient curve did not increase resolution of the enzyme from the inactive protein.

Figure 47. Chromatography of methyltransferase (53-70% alcohol fraction - Table 30) on DEAE-cellulose. The solid line represents optical density at 280 m μ and the shaded curve represents enzyme activity as units per ml effluent. The molarity of the phosphate gradient is shown as a dashed line. A total of 650 mg of protein (2550 units of enzyme) was added to the column (6 x 20 cm), and fractions of 8.8 ml were collected at a rate of 4 ml/min.

Hydroxylapatite Chromatography. Thirty-five ml of the purified enzyme from the previous step were lyophilized, reconstituted in 8.0 ml distilled water and dialyzed in 2 liters of 0.01 M phosphate buffer, pH 6.8. Lyophilization and dialysis did not result in any loss of activity, and the lyophilized material could be stored up to one year with no apparent loss in activity. The dialyzed enzyme containing 16 mg protein and 245 units of activity was added to a 1 x 20 cm column of hydroxylapatite. Elution was carried out with three changes of buffer at a rate of 0.25 ml/minute, and fractions of 3.0 ml were collected. A typical experiment is shown in Figure 48. It may be seen that inactive protein is removed with the first two buffers, and the bulk of the enzyme is eluted with 0.1 M phosphate buffer. Approximately 20% of the added enzyme is recoverable with a specific activity over 500-fold that of the original toluene extract (Table 30). Although these samples were used successfully in a number of experiments to be

presented below, the major problem with the hydroxylapatite eluates was stability. No convenient way was found to stabilize the samples and, in any case, the small amount of material available made further experiments difficult. Therefore, other methods of purification were investigated.

Figure 48. Chromatography of methyltransferase (DEAE-cellulose eluate - Table 30) on hydroxylapatite. The solid line represents optical density at 280 m μ and the shaded curve represents enzyme activity as units per ml effluent. A total of 16 mg of protein (245 units of enzyme) was added to the column (1 x 20 cm), and fractions of 3.0 ml were collected at a rate of 0.25 ml per min.

Batch Treatment with DEAE-Cellulose. It may be seen in Figure 47 that the purification achieved during DEAE-cellulose chromatography is due mainly to the removal of inactive protein with 0.02 M phosphate buffer. Therefore, it seemed possible to achieve a similar result with a batch treatment. The results of the procedure developed are illustrated in Table 31. Twenty-five ml of enzyme (53-70% alcohol fraction) were adjusted to 0.055 M phosphate with 0.2 M phosphate buffer, pH 6.8. This solution, containing 720 mg of protein and 2540 units of activity, was made into a slurry by the addition of 1 mg DEAE-cellulose (previously prepared) per mg protein. After stirring at 1° for 30 min, the mixture was filtered through a Millipore filter (diameter, 47 mm; pore size, 3 μ) and was washed with 5 ml aliquots of 0.055 M phosphate buffer, pH 6.8. The filtrate and washings contained 94% of the enzyme originally present (Table 31). This material was desalted by passing it through a 2 x 30 cm column of Sephadex G-25 using distilled water as the eluting agent, and 94% of the enzyme added to the column was recovered (Table 31). Five mg DEAE-cellulose per mg protein were then added to the desalted filtrate. After 30 min at 1°, the material was filtered through a Millipore filter as above. The

cellulose was washed with two 10-ml aliquots of 0.015 M phosphate buffer, pH 6.8, followed by three 15-ml aliquots of 0.2 M phosphate buffer, pH 6.8. The 0.2 M eluates contained 37% of the original activity of the alcohol fraction with a 3.4-fold increase in specific activity (Table 31). These results compare favorably with DEAE-cellulose column chromatography as a means of purification (see Table 30). The important advantages are the speed with which this may be carried out (3-4 hr) and the possibility of preparing large amounts of material in rather concentrated form. In experiments similar to that cited in Table 31, 25% of the enzyme was obtained in a 15 ml volume of 0.2 M phosphate buffer. Enzyme purified in this manner was dialyzed in 0.01 M phosphate buffer, pH 6.8, and placed on a hydroxylapatite column with results similar to those shown in Table 30 and Figure 48.

TABLE 31

Alternate method of purification of methyltransferase

Treatment	Total protein, mg	Total activity, units	Yield, %	Specific activity, units/mg protein	Purification factor*
Alcohol fractionation	720	2540	100	3.5	21
0.055 <u>M</u> DEAE-cellulose filtrate	460	2380	94	5.2	30
Sephadex G-25 filtrate	430	2230	88	5.2	31
0.2 <u>M</u> DEAE-cellulose filtrate	79	930	37	11.9	70
DEAE-Sephadex chromatography	6.1**	71**	2.8**	11.7	69
Sephadex G-75 chromatography	0.22**	18**	0.7**	82.3	480

*Based on a specific activity of 0.17 for toluene crude extract (see Table 30).

**These values were obtained with 1/7 of the 0.2 M DEAE-cellulose filtrate.

DEAE-Sephadex Chromatography. Seven ml of the solution eluted by 0.2 M phosphate buffer from DEAE-cellulose (Table 31) were dialyzed against 500 ml of 0.02 M phosphate - 0.05 M NaCl, pH 7.0. The dialyzed product, containing 11.2 mg of protein and 133 units of enzyme in 8.0 ml, was added to a DEAE-Sephadex column at a rate of 1 ml per 15 min. The results of a typical experiment are shown in Table 31 and Figure 49. It may be seen that 55% of the added enzyme was eluted with 1.0 ml of 0.02 M phosphate - 0.2 M NaCl, pH 7.0 (Table 31). In similar experiments with enzyme of low specific activity (e.g., 53-70% alcohol fraction) a purification of 4- to 5-fold was obtained. This technique was primarily valuable for the concentration of enzyme solutions with high activity but low protein content. Although dry Sephadex G-25 was used successfully to concentrate enzyme solutions containing 20-30 mg protein per ml, it was

ineffective for solutions containing less than 1 mg protein per ml. In such cases DEAE-Sephadex in short columns was most effective.

Figure 49. Chromatography of methyltransferase (0.2 M DEAE-cellulose filtrate - Table 31) on DEAE-Sephadex. The solid line represents optical density at 280 mμ and the shaded curve represents enzyme activity as units per ml effluent. A total of 11.2 mg of protein (133 units of enzyme) was added to the column (0.5 x 13 cm) and fractions of 1.0 ml were collected at a rate of 1.0 ml per 15 min.

Sephadex G-75 Chromatography. The active eluate from the DEAE-Sephadex column (containing 6 mg of protein and 71 units of enzyme in 1.0 ml) was added to the 0.9 x 200 cm column of Sephadex G-75 equilibrated with 0.2 M phosphate buffer, pH 7.0. The sample was washed into the column with three 1.0 ml aliquots of the same buffer. Subsequently, a 2-liter feeder bottle containing the same buffer was attached, and eluate fractions of 2.2 ml were collected at a flow rate of 8.0 ml per hour. The results of one experiment are shown in Figure 50 and Table 31. Forty per cent of the enzyme emerged after the major protein peak in the two peak tubes. Twenty-five per cent of the enzyme was obtained in one tube with a specific activity 7 times that of the material placed on the column or 500 times the specific activity of the original toluene extract. The final product was as active as the hydroxylapatite fractions (Figure 48), but in contrast to the latter, the enzyme was more concentrated and much more stable and could be stored frozen for several months with no appreciable decrease in activity. It is interesting to note that 99% of the protein and 85% of the total activity were recovered (Figure 50). Attempts were made to achieve further purification with a second Sephadex G-75 column or a Sephadex G-100 column, but there was no increase in specific activity. Similarly,

further purification of the Sephadex G-75 eluates by chromatography on hydroxylapatite columns or purification of the hydroxylapatite eluates on Sephadex G-75 columns has not yet been achieved.

Figure 50. Chromatography of methyltransferase (DEAE-Sephadex concentrate - Table 31) on Sephadex G-75. The solid line represents optical density at 280 mμ and the shaded curve represents enzyme activity as units per ml effluent. A total of 6 mg of protein (71 units of enzyme) was added to the column (0.9 x 200 cm) and fractions of 2.2 ml were collected at a rate of 8.0 ml per hr.

Analysis of Reaction Products. In order to gain insight into the mechanism of the reaction, an analysis of the products was carried out after incubation of reaction mixtures with labeled substrates. The results of a typical series of experiments are summarized in Table 32. Equimolar quantities of both products were produced in all cases. However, significant radioactive methionine was found only when homocysteine-S³⁵ or S-adenosylmethionine-C¹⁴H₃ served as substrates. In each case the specific activity was virtually identical with that of the corresponding substrate. Under the same conditions the S-adenosylhomocysteine produced was not radioactive. On the other hand, with S-adenosylmethionine labeled with S³⁵ or 2-C¹⁴, the S-adenosylhomocysteine had the same specific activity as the methyl donor, whereas the methionine was inactive.

Specificity of the Reaction. No methyl acceptor other than homocysteine has been demonstrated with the purified enzyme. Although cystathionine substitutes for homocysteine in the biosynthesis of methionine with extracts of *A. aerogenes* and *E. coli*, (14) the compound is inactive even with crude toluene extracts.

TABLE 32

Analysis of reaction products

Standard reaction mixtures with each labeled substrate were prepared in a volume of 2.0 ml. After incubation for 2 hr at 37°, the products were isolated and the yield and specific activity were determined in each case as described in the experimental procedure.

Substrate *	Products formed			
	Methionine		S-Adenosylhomocysteine	
	Yield, μmoles/ml	% Incorporation of label	Yield, μmoles/ml	% Incorporation of label
AM-C ¹⁴ H ₃	1.35	98	1.28	2
AM-S ³⁵	1.30	2	1.35	97
AM-2C ¹⁴	1.28	8	1.35	94
Homocysteine-S ³⁵	1.25	98	1.30	3

*The specific activities of the substrates used were, in cpm/μmole: AM-C¹⁴H₃, 91,000; AM-S³⁵, 64,000; AM-2C¹⁴, 50,000 and homocysteine-S³⁵, 100,000.

The only methyl donor that substitutes for S-adenosylmethionine is S-methylmethionine. The ratio of activity of the two methyl donors remains unchanged as the enzyme is purified (Table 30), suggesting that only one enzyme is involved. Among the inactive methyl donors are the sulfonium compounds, acetothetin, propiothetin, and S-ribosylmethionine.

Properties of the Enzyme. The reaction catalyzed by S-adenosylmethionine: homocysteine methyltransferase proceeds according to the equation:

The formation of methionine continues at a linear rate only if the concentration of methionine is less than 0.5 μmole per ml reaction mixture.⁽¹⁾ However, the reaction proceeds to completion with concentrations of 0.1 to 0.2 μmole per ml of methyl donor. It has been impossible to demonstrate reversal of the reaction by incubation of the enzyme with the products. Similarly, no significant incorporation of label could be detected in S-adenosylmethionine after incubation of standard reaction mixtures with unlabeled substrates and 0.1 μmole of methionine-C¹⁴H₃ (10⁵ cpm).

The homocysteine methyltransferase exhibits optimum activity between pH 7.5 and 8.0 (Figure 51). The curves with both methyl donors are almost identical. Routine assays for enzyme activity were carried out at pH 7.0 since the methyl donors undergo chemical decomposition above this pH.

Figure 51

Activity of methyltransferase as a function of pH of reaction mixture. Standard assays were performed except that S-adenosylmethionine- $C^{14}H_3$ was used at a concentration of $0.4 \mu\text{mole per } 0.5 \text{ ml reaction mixture}$ and the specific activity of the methyl donor was $125,000 \text{ cpm}/\mu\text{mole}$. Phosphate buffer (0.2 M) was used for the pH values of $5.0-7.5$; veronal-HCl buffer (0.1 M) for pH values above 7.5 .

The addition of boiled yeast extract or concentrated dialysates of crude enzyme to reaction mixtures causes no appreciable increase in activity of purified, dialyzed enzyme. It appears, therefore, that no easily dissociable coenzyme is present. As shown in an earlier report, (15) inhibition of enzyme activity by chelating agents is reversed by various metals, dialysis, or dilution. This suggests the participation of a metal in the reaction. However, after extensive dialysis of the highly purified enzyme, it is not possible to demonstrate an absolute metal requirement. It appears, therefore, that there is a very firm metal-enzyme bond.

Discussion

The biosynthesis of methionine from homocysteine may proceed via the addition of an intact methyl group or by de novo synthesis of the methyl group. In yeast cells, only the former reaction has been clearly established. Therefore, the enzyme described in this paper, S-adenosylmethionine: homocysteine methyltransferase, carries out the terminal step in the biosynthesis of methionine in yeast cells. Net synthesis of methionine would occur if S-adenosylhomocysteine were regenerated to S-adenosylmethionine by de novo synthesis of a methyl group or by transmethylation. Evidence for regeneration in whole yeast cells was reported by Duerre and Schlenk⁽¹⁶⁾ and by Pigg et al.⁽¹⁷⁾ A cell-free preparation that catalyzes this reaction has not yet been obtained. Therefore the origin of the methyl group and the mechanism of the reaction are unknown. One may speculate that S-adenosylhomocysteine may serve as an acceptor in de novo synthesis of the methyl group in a manner similar to the mechanism established for homocysteine with E. coli extracts.^(3,4)

The homocysteine methyltransferase of yeast cells utilizes S-adenosylmethionine and S-methylmethionine as methyl donors. The same enzyme seems to be involved, because the relative activity with these substrates remains constant during a variety of purification steps. The enzyme has also been found in A. aerogenes, E. coli and rat liver.⁽¹⁾

The homocysteine methyltransferase is the only methyltransferase yet described that seems to require metal ions for its activity. In contrast to the complex of cofactors required for de novo synthesis of the methyl group of methionine,^(3,4) this enzyme seems to have no demonstrable cofactor requirement.

It is clear that S-adenosylmethionine plays an important role in the biosynthesis of methionine via transmethylation. Recent experiments⁽⁴⁾ have indicated that the sulfonium compound is also required, although in catalytic amounts, in de novo synthesis of the methyl group of methionine. Thus, it appears that S-adenosylmethionine has a vital function in both known pathways of methionine biosynthesis.

Summary

The S-adenosylmethionine:homocysteine methyltransferase of S. cerevisiae has been purified over 500-fold. No cofactors have been demonstrated, but participation of a metal is indicated. The general properties of the enzyme are described. The reaction has been shown to involve only the transfer of the methyl group of S-adenosylmethionine to homocysteine, resulting in the products methionine and S-adenosylhomocysteine.

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THE CALCULATION OF ISOTOPE SEPARATION FACTORS OCCURRING IN THE CHROMATOGRAPHY OF DUAL-LABELED COMPOUNDS

Donald W. Simborg* and Peter D. Klein

One of the attractive potentialities of double isotope techniques is the positive identification of an unknown member of a class of compounds by co-chromatography of a tritium-labeled derivative of the unknown with the radiocarbon derivative of the suspected known compound. We have found that the anticipated criterion of a constant isotope ratio across the mixed peak was not fulfilled even when the same compound was used to prepare both derivatives. Specifically, the co-chromatography of cholesterol acetate-1-C¹⁴ with cholesterol acetate-2-H³ resulted in two overlapping, but distinctly displaced curves for each form of radioactivity; that is, isotopic fractionation had occurred in the chromatographic process. Such an occurrence is not entirely unexpected in light of previous experiences with inorganic ions, (1) small organic molecules (2) and labeled amino acids (3) on ion exchange columns, but in contrast to single radioisotope-stable isotope fractionations, which depend upon specific activity measurements, the fractionation of one radioisotope from another is indicated by changes in the ratio of radioisotopes and is, therefore, more easily detected and measured.

Two consequences of this effect are apparent: first, that the dual-isotope technique may still permit the establishment of identity originally sought, provided that the magnitude and sign of displacement of one isotope from the other is known for the two derivatives; and second, that by providing two labels whose relative positions in the molecule can be altered, the technique offers the possibility of mapping the effect of position on the degree of separation and thereby could elucidate intramolecular contributions to the adsorption process. Both of these consequences, however, require accurate knowledge of the degree of displacement or separation factor.

This report deals with an improved procedure for the calculation of separation factors, specifically designed for dual-labeled experiments, which eliminates several conditions required in previous calculations of this type.

Several treatments of the relationship between specific activity and fraction number have been reported. Piez and Eagle (3) have shown that the equation

$$\ln S = \frac{(m_1 - m_2) x}{\sigma^2} + \frac{m_2^2 - m_1^2}{2\sigma^2}, \quad (1)$$

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where S is the specific activity, m_1 is the mean of the activity curve, m_2 is the mean of the colorimetric curve, σ is the standard deviation and x is the fraction number, will predict the specific activity curve in the region of overlap between the two peaks if the distributions are assumed to be identical.

Glueckauf⁽⁴⁾ has provided a specific solution for chromatographic columns by considering the proportion of material eluted in a given segment of the elution curve. From this, he obtains for the ratio of isotopes the following relationship:

$$C_1/C_2 = (m_1/m_2) / (\bar{v}_1/\bar{v}_2) \exp \left\{ \frac{N}{2} \left[\frac{(\bar{v}_2 - v)^2}{\bar{v}_2 v} - \frac{(\bar{v}_1 - v)^2}{\bar{v}_1 v} \right] \right\} \quad (2)$$

where C_1, C_2 are the concentrations of isotopes 1 and 2 in the fraction

m_1, m_2 are the amounts of material applied to the column
 $\bar{v}_1, \bar{v}_2$ are the peak elution volumes for isotopes 1 and 2
 N is the number of plates generated in the column
 v is the elapsed volume of eluent.

The local separation factor δ is defined as the separation factor minus one; the latter is given by the ratio of $\bar{v}_2/\bar{v}_1$. The center of the mixed peak, $\tilde{v}$, is obtained from

$$\tilde{v} = \sqrt{\bar{v}_1 \bar{v}_2} \quad (3)$$

This leads to the transformation:

$$\ln (C_1/C_2) / (m_2/m_1) = \ln (C_1/C_2) / (C_2^0/C_1^0) \cong N\delta \frac{\tilde{v} - v}{\sqrt{v\tilde{v}}} \quad (4)$$

N has been calculated by Glueckauf according to the formula:

$$N = 8(\bar{v}/\beta)^2 \quad (5)$$

where β is the bandwidth at the point where $C_{\max}$ has fallen to $1/e$ of its original value.

The gradient $N\delta$ is obtained from a plot of $\ln (C_1/C_2) / (C_2^0/C_1^0)$ against $\tilde{v} - v / \sqrt{v\tilde{v}}$ or against the approximation $\tilde{v} - v / \tilde{v}$, and from knowledge of N , δ is obtained.

Although this treatment represents an accurate means of obtaining the local separation coefficient, it leaves undefined the error contribution of the plate number and moreover requires the assumption that the dispersion of the two isotopes is identical or nearly so. While this assumption is met in most cases, particularly those involving a single radioisotope, the

utility of any such calculation is increased when it becomes possible to recognize the presence of, as well as correct for, differences in dispersion. The enabling equations will be presented below.

We have found it convenient to use both synthetic and analytical approaches in a complementary fashion through the use of an IBM 1620 to a) construct synthetic chromatograms and isotope ratio curves from known parameters and b) compute the relationships derived analytically for comparison. In all instances, complete agreement was attained.

The synthetic chromatogram program has the following properties: given the number of counts of each isotope, n_1 and n_2 , their respective mean elution volumes M_1 and M_2 , and dispersions σ_1 and σ_2 , as well as the fraction size Δx , the program calculates an ideal (i.e., Gaussian) elution curve for each isotope, its concentration in each fraction, and the isotope ratio ϕ of the fraction. A complete description of the program and its applicability to three-component systems is provided elsewhere. (5)

Behavior of the Isotope Ratio ϕ with Simple Displacement

A series of synthetic chromatograms was obtained for constant n_1 , n_2 , σ_1 , σ_2 and M_1 , varying only M_2 . The behavior of the isotope ratio ϕ is shown in Figure 52. With small displacements (ΔM), ϕ is approximately linear with x , the fraction number, but soon displays a curvature as ΔM reaches 1% of M_1 . Progressive analysis at constant M_2 for various σ_1 , σ_2 and for various n_1 , n_2 , resulted in the equation:

$$\phi = (n_1/n_2) \exp \left[\frac{(M_2 - M_1)}{\sigma^2} (\bar{M} - x) \right] \quad (6)$$

where σ^2 is the product $\sigma_1\sigma_2$ when $\sigma_1 = \sigma_2$, $\bar{M}$ is the arithmetic average of M_1 and M_2 and x is the fraction number.

Figure 52

Behavior of the isotope ratio ϕ for various displacements of M_2 from M_1 , for two chromatographic peaks having the characteristics of n counts, dispersion σ and elution volume M shown. ϕ is the ratio of the first isotope to the second.

When the same parameters are used to construct a synthetic chromatogram and to calculate ϕ according to Equation 6, the resulting isotope ratio curves coincide completely, as shown in Figure 53.

Figure 53

Lower graph: Synthetic chromatogram constructed by machine program for two peaks having the characteristics listed. Upper graph: the ratio of isotope I to isotope II. Circles, derived from the synthetic chromatogram; crosses, computed from Equation 6.

Behavior of the Isotope Ratio ϕ with Changes in Dispersion of Zero Displacement

Figure 54 displays the effect of a difference in dispersion (σ) on the isotope ratio for two chromatographic peaks with identical retention

Figure 54

Behavior of the isotope ratio ϕ (counts of isotope I per counts of isotope II for two peaks having zero displacement but different dispersions).

volumes ($M_1 = M_2$). This can be predicted from the individual equations for the distribution function

$$\begin{aligned} f(x) &= \frac{1}{\sqrt{2\pi}\sigma_1} \exp\left[\frac{-(x - \mu_1)^2}{2\sigma_1^2}\right] dx \\ f(x) &= \frac{1}{\sqrt{2\pi}\sigma_2} \exp\left[\frac{-(x - \mu_2)^2}{2\sigma_2^2}\right] dx \end{aligned} \quad (7)$$

where μ_1 and μ_2 are the respective means (or retention volumes) and x is the fraction number.

Dividing and rearranging, one obtains:

$$\phi = \sigma_2/\sigma_1 \exp\left[\frac{-\sigma_2^2(\mu_1 - x)^2 + \sigma_1^2(\mu_2 - x)^2}{2\sigma_1^2\sigma_2^2}\right] \quad (8)$$

For small differences between μ_1 and μ_2 , $(\mu_1 - x)$ and $(\mu_2 - x)$ may be replaced by $(\bar{M} - x)$,

$$\phi = \sigma_2/\sigma_1 \exp\left[\frac{\sigma_1^2 - \sigma_2^2(\bar{M} - x)^2}{2\sigma_1^2\sigma_2^2}\right] \quad (9)$$

The coincidence of values calculated from this equation and those obtained from the synthetic chromatograms is shown in Figure 55.

Figure 55

Lower graph: Synthetic chromatogram constructed by machine program for two peaks having the characteristics listed. Upper graph: Isotope ratio. Circles, synthetic values from chromatogram; crosses, computed from Equation 9.

Behavior of the Isotope Ratio ϕ for Simultaneous Variations in Retention Volume, Dispersion and Counts

Combining Equations 6 and 9, the over-all equation for any two compounds 1 and 2, with retention volumes M_1 and M_2 , counts n_1 , n_2 and dispersions σ_1 , σ_2 is:

$$\phi = (n_1/n_2) / (\sigma_2/\sigma_1) \exp \left[\frac{(M_2 - M_1)}{\sigma_1 \sigma_2} (\bar{M} - x) + \frac{\sigma_1^2 - \sigma_2^2}{2\sigma_1^2 \sigma_2^2} (\bar{M} - x)^2 \right] \quad (10)$$

An example of the synthetic and analytical values for a particular set of parameters is given in Figure 56.

Figure 56

Lower graph: Synthetic chromatogram for two compounds having different amounts of activity, with differences in dispersion σ and elution volume M . Upper graph: Isotope ratio. Circles, synthetic values from chromatogram; crosses, computed from Equation 10.

Calculation of ΔM and Its Standard Error

If the cumulative per cent of each isotope eluted is calculated for each fraction of the peak, a probit analysis(6) will yield the following quantities: $x_{50\%} \equiv M_1$, M_2 , the fraction at which 50% of the isotope has been eluted; $S.E.M$, the standard error of the retention volume M_1 or M_2 ; σ , the dispersion or standard deviation of the distribution; and $S.E._\sigma$, the standard error of the dispersion. These quantities, together with the ϕ values for the corresponding fractions permit three estimates to be made of ΔM and its standard error:

I. Probit Value

$$\Delta M = M_2 - M_1; S.E.\Delta M = \left[(S.E.M_1)^2 + (S.E.M_2)^2 \right]^{1/2} \quad (11)$$

II. Ln- ϕ

If the dispersions are identical, then Equation 6 may be used, in the form:

$$\ln \phi = \frac{(M_2 - M_1)(\bar{M})}{\sigma_1 \sigma_2} + \frac{(M_2 - M_1) \times}{\sigma_1 \sigma_2} \quad (12)$$

A linear regression analysis of $\ln \phi$ vs x yields the slope, a , and its standard error, $S.E._a$, and represents the quantity $-(M_2 - M_1)/\sigma_1 \sigma_2$, from which multiplication by $\sigma_1 \sigma_2$ yields ΔM .

The standard error of ΔM is given by:

$$S.E._{\Delta M} = \Delta M \left[(S.E._{\sigma_1/\sigma_1})^2 + (S.E._{\sigma_2/\sigma_2})^2 + (S.E._{a/a})^2 \right]^{1/2} \quad (13)$$

III. MODI- ϕ

For two chromatographic peaks of different dispersions, Equation (10) may be used in the form:

$$\left[\ln \left(\frac{n_2 \sigma_1}{n_1 \sigma_2} \right) \phi - \frac{\sigma_1^2 - \sigma_2^2 (\bar{M} - x)^2}{2\sigma_1^2 \sigma_2^2} \right] = \frac{(M_2 - M_1)(\bar{M})}{\sigma_1 \sigma_2} - \frac{(M_2 - M_1)(x)}{\sigma_1 \sigma_2} \quad (14)$$

The method of calculating ΔM and its standard error is then identical to that for $\text{Ln-}\phi$.

In comparing one chromatographic run with another, or one pair of retention volumes with another, it is preferable to have ΔM expressed as a per cent of M_1 . The relationship between $\Delta M\%$ and the conventional separation factor is:

$$\text{Separation factor} = 1 + \frac{\Delta M\%}{100} \quad (15)$$

$$\text{Glueckauf's local separation factor } \delta = \frac{\Delta M\%}{100} \quad (16)$$

Results

Some examples of the application of these calculations are shown in Table 33. The input data were obtained from three chromatographic runs of a mixture of cholesterol acetate-2-C¹⁴ and cholesterol acetate-2-H³ on silica gel columns eluted by 16% benzene in pentane. The per cent displacement ($\Delta M\%$) was calculated by each of the three methods. The component parameters used are listed together with their standard errors. It is evident that both $\text{Ln-}\phi$ and $\text{MODI-}\phi$ represent a more precise form of computation than the probit method since the first two have a much smaller

associated standard error. On the other hand, the standard error of the average value is comparable for the three methods, as are the average values themselves. This indicates that the experimental variation from run to run is as yet larger than the error in computation.

TABLE 33

Comparison of results from probit, $\text{Ln-}\phi$, and $\text{MODI-}\phi$ calculations for
Cholesterol acetate- 2-C^{14} , 2-H^3

Run	S.E.	Probit	S.E.	$\text{Ln-}\phi$	S.E.	$\text{MODI-}\phi$	S.E.
122.1 M_1^*	123.382 0.114						
M_2^{**}	122.961 0.126						
σ_1	6.485 0.119						
σ_2	6.464 0.132						
a (slope)				0.01038	0.00164	0.01167	0.00188
ΔM		-0.421 0.170		-0.435	0.070	-0.489	0.065
$\Delta M\%^{\dagger}$		-0.341 0.138		-0.352	0.057	-0.396	0.053
122.2 M_1	108.986 0.105						
M_2	108.492 0.101						
σ_1	5.941 0.108						
σ_2	5.921 0.104						
a				0.01355	0.00071	0.01403	0.00089
ΔM		-0.494 0.137		-0.477	0.028	0.494	0.034
$\Delta M\%$		-0.453 0.137		-0.438	0.026	0.453	0.031
122.3 M_1	116.812 0.032						
M_2	116.106 0.039						
σ_1	5.458 0.031						
σ_2	5.464 0.038						
a				0.02203	0.00078	0.02415	0.00065
ΔM		-0.706 0.051		-0.657	0.024	-0.720	0.020
$\Delta M\%$		-0.604 0.043		-0.562	0.020	-0.616	0.017
Average $\Delta M\%$		-0.466 0.076		-0.451	0.060	-0.488	0.066

*Retention volume of H^3

**Retention volume of C^{14}

† The isotope ratio ϕ is the ratio of isotope I to isotope II. By the conventional expression of H^3/C^{14} , the displacement has a negative sign, since H^3 emerges after C^{14} .

Comparison of the $\text{Ln-}\phi$ and $\text{MODI-}\phi$ values and their standard errors does not, in these instances, demonstrate the benefits to be expected from the improvement in computation method when $\text{MODI-}\phi$ is

used. This is not unexpected, however, in light of the extremely close agreement between the dispersion values (σ_1 and σ_2), which for all practical purposes are identical.

Table 34 lists the results from two experiments which further illustrate the relationship of the three methods. In the first experiment, the dually-labeled cholesterol formed by in vivo incubation of a mixture of mevalonic acid-2- C^{14} and mevalonic acid-5- H^3 was chromatographed and the segregation of C^{14} -rich cholesterol from H^3 -rich cholesterol was measured. The presence of a contaminant (methostenol) in the tail of the chromatographic peak results in a curvature of the upper portion of the probit line. This curvature, while without significant effect on $\Delta M\%$ calculated by the probit technique, significantly increases the standard error of the measurement. The other two methods, however, are uninfluenced in this instance. It should be noted that although the average values and standard errors again agree entirely with one another, the individual values calculated by the probit method would be regarded as nonsignificant in the absence of other criteria.

TABLE 34

Comparison of results from probit, $\text{Ln}-\phi$, and $\text{MODI}-\phi$ calculations

Run	I. Cholesterol-x- C^{14} -y- H^3 acetate				
	Probit	S.E.	$\text{Ln}-\phi$	S.E.	$\text{MODI}-\phi$ S.E.
34.1	-0.208	0.271	-0.238	0.040	-0.237 0.040
34.2	-0.245	0.221	-0.268	0.015	-0.230 0.018
34.3	-0.267	0.270	-0.276	0.020	-0.219 0.023
Average	-0.240	0.017	-0.261	0.010	-0.228 0.010

Run	II. Dihydrolanosterol-24, 25-di- H^3 acetate-1- C^{14}				
	Probit	S.E.	$\text{Ln}-\phi$	S.E.	$\text{MODI}-\phi$ S.E.
126.2	-0.0012	0.0777	-0.1210	0.0402	+0.0071 0.0236
126.3	-0.1770	0.1324	-0.2591	0.1325	-0.1080 0.0666
126.4	-0.0650	0.1540	-0.0716	0.0404	-0.0019 0.0262
Average	-0.0616	0.0580	-0.1143	0.0746	-0.0343 0.0370

The second experiment in Table 34 is an example of the values obtained when isotopic fractionation is absent. The values are characterized in all three methods by a standard error which approaches or exceeds the value of the individual $\Delta M\%$ as well as of the average $\Delta M\%$. This illustrates that all three methods will indicate nonsignificant displacement when fractionation is absent.

Discussion

The three methods of calculation presented here form a progressive series in which the second method builds upon the first and the third upon the first and second. The potential contribution of probit analysis does not appear to have been widely utilized in prior computations despite its relative simplicity; this may reflect the tediousness of the hand calculation involved before machine programs became available. Once obtained, however, the probit analysis provides a reliable approximation of the displacement between the two isotopes as well as a basis for more precise calculations, in that it provides the required estimate of the two means and their dispersions.

The equation for the behavior of the isotope ratio in the absence of different dispersions ($\text{Ln}-\phi$) can be shown to be identical to the two prior forms described by Piez and Eagle⁽³⁾ and by Glueckauf.⁽⁴⁾ This identity with prior derivations is further confirmation of the validity both of the present equations and of the synthetic approach used to obtain them.

Both $\text{Ln}-\phi$ and MODI- ϕ introduce improvement over previous methods by providing error estimates for the values obtained. In addition, the MODI- ϕ equation describes the effect of differences in dispersion upon the isotope ratio, permits the calculation of true displacement in the presence of such differences and thereby facilitates the comparison of any two compounds which migrate at closely similar rates. This comparison makes it possible to carry out the chromatographic determination of identity originally sought for the dual-labeled acetates. The MODI- ϕ equation also points up the unexpected finding that isotopic enrichment is possible in the absence of displacement if there is a significant difference in the dispersions of the two forms.

Finally, the existence and availability of machine programs for these calculations should be emphasized since these programs greatly reduce the time and effort necessary to translate chromatographic data into displacement values. In these circumstances the fractionation of one isotope from another can be used as an analytical parameter in the study of intramolecular contributions to the adsorption process.

Summary

Three equations for the calculation of isotope fractionation values have been examined. These are: the probit analysis, which measures the simple displacement of one peak from another; $\text{Ln}-\phi$, the relationship of isotope ratio to the displacement of one peak from another where both dispersions are identical; and MODI- ϕ , the relationship of isotope ratio to displacement of one peak from another where the dispersions are different. The MODI- ϕ method of calculation provides the basis for a broader usage of isotope fractionation in the study of the chromatographic process.

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A COMPUTER PROGRAM TO SIMULATE A TWO-COMPONENT CHROMATOGRAM WITH OR WITHOUT CONTAMINANT

Barbara Kunze,* Sylvia Tyler,** and Merlin H. Dipert

A study of isotope fractionation during chromatography of dual-labeled compounds(1) has been greatly expedited by a procedure for constructing synthetic chromatograms from known parameters for the means, standard deviations and numbers of counts, using the assumption that the distributions are Gaussian. The Fortran program was designed for the IBM-1620 to assist in a study of the separation of dual-labeled tritium and carbon materials with and without a contaminant. The program makes it possible to consider two or three overlapping normally distributed populations: to find the number of counts for each component within an interval, and to compute their ratios and the cumulative percentages of total counts for the individual curves. A Fortran listing of the program is given in Figure 57 and a block diagram in Figure 58.

To compute the number of counts in the interval, Hastings' approximations(2) are used. It is desired to compute the area under the normal probability curve for a finite interval, using t as a dummy variable:

$$\frac{1}{\sqrt{2\pi}} \int_x^\infty e^{-t^2/2} dt = \frac{1}{2} - \frac{1}{2} \Phi\left(\frac{x}{\sqrt{2}}\right)$$

$$\Phi(\alpha) = \frac{2}{\sqrt{\pi}} \int_0^\alpha e^{-t^2} dt; \quad 0 \leq \alpha < \infty.$$

Using Hastings' approximations

$$\Phi(x) \approx 1 - \frac{1}{[1 + A_1\alpha + A_2\alpha^2 + A_3\alpha^3 + A_4\alpha^4 + A_5\alpha^5 + A_6\alpha^6]^{16}}$$

$$a_1 = .070523078$$

$$a_4 = .0001520143$$

$$a_2 = .042282012$$

$$a_5 = .0002765672$$

$$a_3 = .0092705272$$

$$a_6 = .0000430638$$

The maximum error is .0000003.

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**Student Aide from Fisk University, Summer 1963.

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C  DISTRIBUTION OF THREE NORMAL POPULATIONS -KUNZE AND TYLER

55  DIMENSION  B(3),S(3),EN(3),C(3),SUMC(3),CP(3),A(2),AA(2),AB(2)
3   READ 3, XO,XL,DELX
   FORMAT (E10.3, E10.3, E10.3)
4   DO 4 I=1,3
   READ 5,B(I),S(I),EN(I)
5   FORMAT (E14.7, E14.7, E14.7)
   DO 10 I = 1,3
   C(I)=0.
10  SUMC(I) = 0.
   N=(XL-XO)/DELX
   DO 60 K=1,N
   DO 80 J=1,3
   XI=K
   A(1)=((XO+(XI-1.)*DELX-B(J))/S(J))/(2.**.5)
   A(2)=((XO+XI*DELX-B(J))/S(J))/(2.**.5)
   DO 17 I =1,2
   AB(I)=(A(I)**2)**.5
17  AA(I)=1.+0.070523078*AB(I)+.042282012*AB(I)**2+.0092705272*AB(I)**3
   AA(I)=AA(I)+.0001520143*AB(I)**4+.0002765672*AB(I)**5
   AA(I)=.5/(AA(I)+.0000430638*AB(I)**6)**16
   IF  (A(1)) 19,20,20
20  C(J)=(AA(1)-AA(2))*EN(J)
   GO TO 90
19  IF  (A(2)) 21,22,22
21  C(J)=(AA(2)-AA(1))*EN(J)
   GO TO 90
22  C(J)=(1.-AA(1)-AA(2))*EN(J)
90  SUMC(J)=SUMC(J)+C(J)
80  CP(J) =SUMC(J) *100./FN(J)
   R1=C(1)/C(2)
   R2 =100.*(SUMC(1)+SUMC(3))/(EN(1)+EN(3))
   R3 = (C(1)+C(3))/C(2)
   C(1) = C(1)+.5
   C(2) =C(2)+.5
   C(3) = C(3) +.5
71  PUNCH 71, K, C(1),C(2),CP(1), CP(2), R1
   FORMAT (I5,F7.0,F7.0,7X,F6.2,F6.2,12X,F8.3)
   IF(SENSE SWITCH 1)35,36
35  PUNCH72, K, C(1), C(3), CP(1), CP(3), R2
72  FORMAT (I5, F7.0, 7X, F7.0,F6.2,6X,F6.2, F6.2)
36  IF(SENSE SWITCH 2)37,60
37  PUNCH 73, K, C(1), C(2), C(3), CP(2), R2, R3
73  FORMAT (I5, F7.0,F7.0, F7.0, 6X, F6.2, 6X, F6.2, 8X, F8.3)
60  CONTINUE
   GO TO 55
   END

```

Figure 57. Fortran listing of the program

Figure 58. Block diagram of the program. Distribution of three normal populations.

In the input to the program, the first card is:

Columns

1-10	XO	(Starting point of interval)
12-20	XL	(End point of interval)
21-30	DELX	(Increment for X)

The next three cards give information about populations 1, 2, and 3, respectively.

Columns

1-14	$\bar{X}$	(Population mean)
15-28	σ	(Population standard deviation)
29-42	N	(Population size)

The output consists of:

1. Interval number
2. Number of first population in interval
3. Number of second population in interval
4. Cumulative per cent of first population
5. Cumulative per cent of second population
6. Ratio: No. of first population in interval No. of second population in interval.

With sense switch 1 ON, a second card is obtained.

1. Interval number
2. Number of first population in interval
3. Number of third population in interval
4. Cumulative per cent of first population
5. Cumulative per cent of third population
6. Cumulative per cent of first and third population

With sense switch 2 ON, a card is obtained with

1. Interval number
2. Number of first population in interval
3. Number of second population in interval
4. Number of third population in interval
5. Cumulative per cent of second population
6. Cumulative per cent of third population
7. (Pop 1 + Pop 2)/Pop 3.

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EFFECT OF X-RADIATION ON OXIDATIVE PHOSPHORYLATION IN RAT LIVER MITOCHONDRIA

John F. Thomson and Linda F. Bordner

Recent studies from this laboratory have indicated that the apparent defect in oxidative phosphorylation induced in spleen or thymus by total-body ionizing radiation is most likely an artifact resulting from inhomogeneity of the mitochondrial preparation.^(1,2) When experimental conditions are chosen that permit efficient phosphorylation in mitochondria isolated from spleens or thymuses of control animals (i.e., P/O ratios of 1.8 to 2.0 with succinate), mitochondrial preparations from tissues of irradiated animals will also efficiently esterify phosphate. When low P/O ratios are observed with control mitochondria, still lower ratios will be obtained after irradiation. The low ratios are probably attributable to the presence of extraneous non-mitochondrial phosphatases of lysosomal or microsomal origin, which become more active after total-body irradiation as a result of endocrine influences.⁽³⁾

According to a recent report,⁽⁴⁾ the capacity of rat liver mitochondria to esterify inorganic phosphate was significantly reduced 3 to 12 hours after 840 r of total-body X-irradiation. These authors used pyruvate, citrate, β -hydroxybutyrate, and glutamate as substrates, and they prepared their mitochondria by conventional differential centrifugation. Their observations are not in agreement with those reported from this laboratory; Thomson and Rahman⁽⁵⁾ observed no impairment of phosphorylation, with succinate or fumarate as substrates, in mitochondria prepared by gradient centrifugation of livers of rats given 1000 r of X-radiation 1 to 24 hr previously.

This report is concerned with additional observations on rat liver mitochondria, with α -ketoglutarate and fumarate as substrates. By means of density-gradient centrifugation, we have prepared and tested, from each liver, four mitochondrial preparations differing in size and homogeneity. Only marginal effects of radiation have been observed.

Methods

Holtzman female rats, two to four months old, were used. The rats were fasted 20 to 24 hr before they were killed. Irradiated rats received 1000 r total-body X-radiation at a dose rate of 200 r/min, and were killed $3\frac{1}{2}$ to 4 hr after exposure.

About 2.5 g of liver were homogenized in 9 volumes of 0.25 M sucrose containing 0.0003 M EDTA. Five-ml aliquots were layered over a sucrose density gradient (45 ml, 10 to 30% sucrose plus EDTA) prepared

24 hr earlier. After centrifugation for 1 hr at 2000 x g, the top 10-ml layer was removed from each tube and discarded. Four subsequent 10-ml portions were removed, and the corresponding layers from each tube were combined and centrifuged at 28,000 x g for 10 min. The sediments (principally nuclei) in the gradient tubes were discarded. The sediments from the four combined layers after centrifugation were resuspended in 2 ml of 0.25 M sucrose (no EDTA) and assayed for oxidative phosphorylation, (1) with either fumarate or α -ketoglutarate as substrates.

In some experiments with α -ketoglutarate, the original homogenate was first centrifuged for 10 min at 600 x g to remove the nuclei. After gradient centrifugation, the layers were removed as before, except that the sediments were resuspended in the bottom layers. The data from the two preparative methods agreed very closely for the first three layers, so that the results of the two sets of experiments with these layers were combined. The data for the bottom layers are tabulated separately; although the phosphate esterification values were similar, the total nitrogen contents were appreciably different.

The ranges of mitochondrial sizes, given as equivalent spherical diameters, were calculated according to Thomson and Klipfel(6) There is actually some overlapping of sizes in different layers since the initial homogenate layer has finite thickness. In addition, because of uncertainties in the value taken for mitochondrial density, the absolute accuracy of the particle sizes is open to question. However, the numbers given are adequate for comparative purposes.

Results and Discussion

It is clear from the data presented in Tables 35 and 36 that there was no consistent effect of X-radiation on either the phosphorylative capacity (P/N ratios) or the phosphorylative efficiency (P/O ratios) of rat liver mitochondria. None of the differences was statistically significant.

Zone A, the uppermost of the four layers studied, is the most inhomogeneous preparation, containing appreciable amounts of lysosomal and microsomal elements; this zone also had the lowest P/O and P/N ratios. However, even in this preparation there were only slight decreases in phosphorylative capacity and efficiency after irradiation, in contrast to what might have been anticipated from our earlier work with rat spleen.(1,2)

At the present time it is difficult to understand why we failed to confirm the observations of Hall et al.(4) These investigators did not include fluoride in their reaction mixtures, whereas we did. It is conceivable that the presence of fluoride minimized the ATPase and ADPase

TABLE 35

Oxidative phosphorylation in mitochondria prepared from livers of control and irradiated rats, with α -ketoglutarate as substrate

Layer*	A	B	C	D ₁	D ₂
Size range (μ)	0.37-0.55	0.55-0.69	0.69-0.82	0.82-0.94	>0.82
Control					
No. rats	11	11	11	7	4
P/N**	18.2 $\pm$ 0.86	27.9 $\pm$ 0.68	29.2 $\pm$ 0.76	30.2 $\pm$ 1.14	33.1 $\pm$ 2.03
P/O†	3.26 $\pm$ 0.13	3.70 $\pm$ 0.09	3.65 $\pm$ 0.08	3.94 $\pm$ 0.14	3.91 $\pm$ 0.17
4 hr after 1000 r					
No. rats	11	11	11	6	4
P/N	17.6 $\pm$ 1.46	26.2 $\pm$ 0.61	28.8 $\pm$ 0.85	28.7 $\pm$ 1.99	28.5 $\pm$ 1.87
P/O	3.01 $\pm$ 0.24	3.86 $\pm$ 0.10	3.82 $\pm$ 0.09	3.85 $\pm$ 0.08	3.65 $\pm$ 0.03

TABLE 36

Oxidative phosphorylation in mitochondria prepared from livers of control and irradiated rats, with fumarate as substrate

Layer*	A	B	C	D ₁
Size range (μ)	0.37-0.55	0.55-0.69	0.69-0.82	0.82-0.94
Control				
No. rats	6	7	7	7
P/N**	12.2 $\pm$ 0.79	15.8 $\pm$ 0.86	17.7 $\pm$ 1.44	13.5 $\pm$ 1.11
P/O†	2.44 $\pm$ 0.13	2.78 $\pm$ 0.13	2.77 $\pm$ 0.06	2.57 $\pm$ 0.12
4 hr after 1000 r				
No. rats	7	6	7	6
P/N	13.1 $\pm$ 0.50	16.8 $\pm$ 1.41	17.8 $\pm$ 0.81	15.9 $\pm$ 1.05
P/O	2.38 $\pm$ 0.19	2.87 $\pm$ 0.15	2.82 $\pm$ 0.18	2.70 $\pm$ 0.19

*D₁, nuclei not removed before layering, sediment not included in bottom layer. D₂, nuclei removed before layering, sediment included in bottom layer.

**Micromoles P esterified/mg N/20 min, averages and standard errors.

†Micromoles P esterified/microatom O consumed/20 min, averages and standard errors.

activities of the liver particulates. However, it was emphasized(1) that the hydrolysis of organic phosphates by preparations of spleen mitochondria was not completely inhibited by the presence of fluoride. Thus an explanation based on the presence or absence of fluoride seems rather remote.

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MOLECULAR AND ULTRASTRUCTURAL ASPECTS OF PLASTID
DEVELOPMENT AND CHLOROPHYLL PRODUCTION
IN THE CHLORELLA MUTANT C-10

Gary W. Bryan and Judy A. Alblinger

Wild-type Chlorella vulgaris produces chlorophyll and develops its photosynthetic apparatus in either the presence or absence of light. C-10, an ultraviolet light-induced mutant, differs from wild-type in its lowered capacity to synthesize chlorophyll in the dark, but resembles higher plants in having the same action spectrum for chlorophyll production.⁽¹⁾ However, when C-10 is grown autotrophically in the light it is indistinguishable from wild-type Chlorella grown under the same conditions. Cell division, chlorophyll synthesis, and chloroplast production go on at the same rate. C-10 also produces chlorophyll in darkness when sufficient levels of iron and glucose are present in the media.⁽²⁾

The problem of interest now is to correlate intracellular structural changes with the molecular transitions induced by light or iron in C-10 (Figures 59-62). From the observations made to date it appears that the

Figure 59. Longitudinal section through a dark-grown cell of Chlorella mutant C-10 nearing the end of its logarithmic growth. Note the irregular shape of the proplastid and the vesicles present within it. P, proplastid; G, Golgi apparatus; Ve, vesicles.

Figure 60. Longitudinal section of a dark-grown cell of Chlorella mutant C-10 that was suspended in distilled water during a 24-hour exposure to 100 foot-candles of fluorescent light (Sylvania Gro-Lux). Note the rigid structure of the newly-developed chloroplast and the lamellae. V, vacuole; C, chloroplast; L, lamellae; Py, pyrenoid; M, mitochondrion.

Figure 61. Cross section through a dark-grown cell of wild-type *Chlorella* nearing the end of its logarithmic growth. Note the rigid structure of the chloroplast and the presence of lamellae. C, chloroplast.

Figure 62. Longitudinal section through a dark-grown cell of wild-type *Chlorella* that was suspended in distilled water during a 24-hour exposure to 100 foot-candles of fluorescent light (Sylvania Gro-Lux). Note the similarity to chloroplasts in Figs. 60 and 61. C, chloroplast.

mutation has blocked the alga's ability in darkness to produce not only chlorophyll but also its entire photosynthetic apparatus: light is required for the formation of lamellae in C-10 as in higher plants. The intermediate stages for this process in the mutant are being examined. The work done to date indicates that for a comparable quantity of chlorophyll produced, the lamellar development induced by iron is similar to that induced by illumination.

Autoradiographs, observed with the electron microscope, of C-10 exposed to light while suspended in tritiated leucine suggest that chloroplast lamellar proteins are synthesized during this period (Figure 63). Despite the poor resolution it can be seen that the newly developed plastid preferentially accumulates the amino acid, a result entirely consistent with that in *Euglena*, in which protein content increases as the plastid develops.⁽³⁾ However, in either cell system, the degree of the interdependence of the structural and the molecular changes upon one another is only partially answered.

Figure 63. Section of dark-grown *Chlorella* mutant C-10 cells that were suspended in tritiated leucine during a 16-hour exposure to red fluorescent light. Ilford L-4 emulsion, 4-day exposure. Note the vermiform silver grains closely associated with the plastid. C, chloroplast; Ag, silver grains.

Since it appears that chlorophyll production is not limited in the C-10 system by the lack of chlorophyll precursors,⁽¹⁾ other factors must be responsible; and one possibility is that the protein of the pigment-protein complex is the limiting factor. More specifically, and consistent with ultrastructural observations of lamellae, it is conceivable that the lattice on which the pigment is placed must be produced before more pigment can be synthesized. In order to test this and related hypotheses, a number of specific experiments are suggested. For example, the use of anti-metabolites to block protein and RNA synthesis should make it possible to define the role of each of these processes more precisely. Also, eight other ultraviolet-induced mutants, each of which resembles C-10 in its limited ability to produce chlorophyll and a chloroplast in the dark, are being observed. Three of them resemble C-10 quite closely, whereas the remainder produce other pigments, primarily carotene, in the light and exhibit varying degrees of structural change.

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A COMPARATIVE STUDY OF GLASS, QUARTZ, AND VYCOR KNIVES FOR ULTRATHIN SECTIONING

Edward W. Daniels, Evelyn P. Breyer,
and Thomas J. Doody

At the present time most electron microscopists use glass or diamond knives for making thin sections of biological material for electron microscopic examination. Since glass knives require considerable time and skill to prepare and, in addition, easily lose their edge, and since diamond knives are expensive, we investigated the possibilities of making durable knife edges from quartz and Vycor.

The discussion below is confined to a comparison of glass, quartz, and Vycor as materials for microtome knives for ultrathin sectioning.

Materials and Methods

Pittsburgh Plate glass ($3/16$ -inch thick) was cut into $1-1/4$ -inch squares and scored along the central part of a slightly off-center diagonal line. The two pieces were separated by gripping opposite edges with pliers and pulling outward and upward (toward the score mark).

Quartz was obtained in $4 \times 4 \times 1/4$ -inch blocks. The surface was cleaned and then etched with an extra-hard Carboloy wheel cutting tool. The blocks were first broken in half along the first etch line, then the 2-inch strips were broken in half lengthwise in a similar manner. Each one-inch strip was then broken in half across the middle, and each of the remaining pieces was again broken in half in a similar manner, giving 16 one-inch squares. The surface of each of these was carefully scored from corner to corner except for 1 to 2 millimeters at the edge. Thus, etch marks on or close to what later became the cutting edge were avoided. By this procedure we were able to obtain significantly more quartz knives with excellent edges than we were with an equivalent amount of glass, broken in a similar series of steps. Knives were broken at various angles, but cutting edges with 40° to 45° gave the best results in sectioned material.

Vycor was obtained in plate form from the Corning Glass Works, Corning, New York and broken in a manner similar to that of quartz.

Cells were embedded in Epon-812 and sections were cut with a Porter-Blum microtome. Sections were examined in an RCA-EMU 3F electron microscope.

Figure 64. Section made with Vycor knife through A. proteus nucleus (N) with surrounding cytoplasm (CY). The smaller micrograph is a section through two Golgi bodies (G). Epon-812 embedded.

Figure 65. Top. Section made with quartz knife through A. proteus nucleus. Center. Section made with glass knife through A. proteus Golgi bodies. Bottom. Section made with glass knife through A. proteus nucleus. Epon-812 embedded.

Results

The three types of knives used in this study were compared in two ways, by microscopic examination of the cutting edges, and by making thin sections. The knife edges were examined with a dissecting microscope at about 50 diameters in reflected light. We found that quartz and Vycor produced long spans of sharp edge, free from serration cracks, at a frequency significantly greater than glass. Vycor and quartz do not age and do not develop fine striae along the cutting edge as glass does.

Our experience in comparing the cutting qualities of these three kinds of microtome knives is limited to the cutting of amoebae (Amoeba proteus) which were fixed in osmium tetroxide and embedded in Epon-812 resin. We sectioned about a dozen different blocks, and cut each block with each type of knife. Vycor knives lasted substantially longer than either glass or quartz and produced uniform sections (ca. 100 m μ) free of knife marks. Figure 64 and inset demonstrate the quality of sections cut with Vycor knives. The former is from a nucleus (N) and the latter is from a section through two Golgi bodies (G). The cytoplasm (CY) is shown around the nucleus. Figure 65 (top) is a section of a nucleus which was made with a quartz knife. Figure 65 (center and bottom) are views of sections made with glass knives. The former was made through two Golgi bodies while the latter is a photograph of the nuclear stratum of a centrifuged Amoeba proteus where the mitochondria (M) are centrifugal to the nucleus.

Vycor is already in use in one laboratory.^(1,2) Our results and conclusions in respect to the superior qualities of Vycor over glass are in agreement with the findings of these investigators, whose work we did not discover until we had completed ours.

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INTRANUCLEAR STRATIFICATION IN THE AMOEBA, PELOMYXA ILLINOISENSIS

Edward W. Daniels, Evelyn P. Breyer,
 and C. Ward Kischer

The cytoplasm of Pelomyxa illinoisensis can be stratified by centrifugation of the whole organism at several thousand times gravity, (1) but the nucleoplasm does not stratify at these forces. Since separation of the nuclear components is also of interest, we attempted to achieve stratification of the nucleoplasm using substantially higher centrifugal forces.

Materials and Methods

The amoebae and procedures for handling them have been previously described.(1,2) Approximately 200 organisms were distributed between two Lusteroid tubes containing a few drops of glycerol to act as a cushion. The tubes were filled to the top with glass distilled water, placed in a 40.2 rotor head, and centrifuged at approximately 1°C in a Model L Spinco ultracentrifuge. The maximum force applied was 140,000 times gravity for 5 min, exclusive of acceleration and deceleration. The amoebae were removed immediately after centrifugation and fixed in 2% cold osmic acid for at least one-half hour, washed in cold Ringer's solution, dehydrated in ethanol and embedded in Epon-812 resin.(3) Sections were made with a Porter-Blum microtome and were examined with an RCA-EMU 3-F electron microscope.

Results

The effects of centrifugation at various forces for different periods of time are shown in Figures 66-69. The nuclei in Figure 66 were centrifuged at 80,000 x g for 15 min. Some stratification can be seen, but the boundary between the electron-dense, centrifugal layer (D) and the less dense (L) portion is not sharp. Some stratification can be seen also in amoebae centrifuged at 133,000 x g for 8 min (Figure 67).

The nuclei of Figures 68 and 69, from amoebae centrifuged at 140,000 x g for 5 min, show a sharp boundary between electron-dense and less dense areas (Figure 68, B). Figure 69 shows an additional stratum (C); it lies between the D and L strata and is the clearest of the three, having almost no large electron-dense particles of the kind seen in the other two. Occasionally stratum C occurred as a sphere surrounded by material from the L stratum at a level above, but adjacent to, the D stratum.

Figure 66. Nuclei of Pelomyxa illinoisensis centrifuged for 15 min at 80,000 x g. The electron-dense components are nearer the centrifugal pole.
N, nucleoli; D, electron-dense component; L, less dense component.

Figure 67. Amoeba nucleus centrifuged at 133,000 x g for 8 min. The boundary between the dense (D) and less dense (L) areas is not sharply defined. Some nucleoli have migrated toward the centrifugal pole.

Figure 68. Amoeba nuclei centrifuged at 140,000 x g for 5 min. The boundary (B) between the dense and less dense strata is sharply defined.

Figure 69

Amoeba nucleus centrifuged at 140,000 x g for 5 min. Note the third stratum (C) which is relatively clear and distinct from the others.

Attempts were also made to stratify the nucleoplasm by centrifugation at 140,000 x g for longer times, at room temperature, and for intermittent intervals (e.g., two of 4 min each). However, the best results were attained by centrifuging in the cold at high centrifugal forces (140,000 x g) for short periods of time (5 min).

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EXPERIMENTS WITH HEAVY METAL COMPOUNDS AS FIXATIVES FOR ELECTRON MICROSCOPY

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Studies on the fine structure of cells, particularly nuclei, have been limited by the extremely small number of good fixatives. Osmium tetroxide (OsO_4) is the best and most widely used general fixative for electron microscopy, while potassium permanganate (KMnO_4) is probably next most frequently used and has been especially valuable for fixing protoplasmic membranes. Both these compounds are powerful oxidizing agents and contain heavy metals, although the electron density of osmium is about 3 times that of manganese.

In an effort to find fixatives with characteristics better than those of either OsO_4 or KMnO_4 , we decided to investigate certain compounds of other elements of about the same density as osmium. We chose platinum, iridium, mercury, and iodine.

We also investigated fixation by the strong oxidizing agent RuO_4 . Ruthenium has a reasonably high density, and RuO_4 has been used as a fixative for light microscope cytology⁽¹⁾ and for reactions with biologically important substances such as DNA, RNA, amino acids, fatty acids, starch, etc.^(2,3)

Materials and Methods

Amoeba proteus were put into the following solutions:

- 1) $\text{Pt}(\text{SO}_4)_2$ (final conc. 0.04 M) in 0.08 M tris(hydroxymethyl)aminomethane, pH 7.0
- 2) IrBr_3 (final conc. 0.04 M) in water
- 3) IrCl_4 (final conc. 0.04 M) in water
- 4) $\text{Ir}_2(\text{SO}_4)_3$ (final conc. 0.04 M) in water
- 5) $\text{Hg}(\text{SO}_3)_2^{--}$ (final conc. 0.02 M) in 0.04 M NaCl
- 6) NaIO_3 (final conc. 0.04 M) in 0.10 M aqueous phosphate buffer, pH 7.0
- 7) I_3^- (final conc. 0.04 M) in 0.10 M phosphate buffer, pH 7.0

- 8) RuO_4 (0.05%) in water
- 9) Solution 8 diluted 1:100 with water

Results

None of the solutions of noble metal compounds were sufficiently toxic to kill the amoebae within 15 min. Consequently, we decided to continue their use as fixatives, at least as used alone in aqueous solution. The mercuric sulfite complex ion preparation killed the cells but failed to fix them in satisfactory form. Nuclei, mitochondria, and other cytoplasmic components were barely recognizable. Essentially similar results were obtained by fixation in 0.04 M aqueous triiodide.

RuO_4 was found to be an extremely fast fixative, more rapid than OsO_4 . Fixation was better in the more dilute of the two solutions used. Evidence of rapid fixation is demonstrated in Figure 70, which shows a pseudopod of Amoeba proteus in an early stage of formation with the plasmasol (PS) separated from the plasmagel (PG). The plasmalemma unit membrane (PM) has been separated from the plasmasol-plasmagel interface (arrows). This membranous type of plasmasol-plasmagel interface has not been seen in any amoeba whose plasmalemma has been raised or separated from the plasmagel by hypotonic solutions following fixation. Thus, it does not appear to be an artifact. Similar pseudopods appear in living amoebae and these can be seen with a phase microscope. A small blister of relatively clear plasmasol forms under the plasmalemma (PL) and this is momentarily separated from the plasmagel which contains the larger formed components of the cytoplasm. The plasmagel (PG) is a temporary boundary and it appears to separate the clear plasmasol in the "blister" (PS) from the central bulk of the amoeba's protoplasm, which is granular and often in the plasmasol state. In reality it might act as a sieve allowing the plasmasol to pass through into the "blister." The RuO_4 fixative is apparently fast enough to stop the sol-gel separation before it reverts to a single state, and the morphology of early pseudopod formation, like that seen in the phase microscope, seems to be preserved. OsO_4 and other fixatives commonly used are not fast enough to produce this result.

In RuO_4 -fixed A. proteus the outer fringe of the plasmalemma (OF in Figure 70) appears as a delicate reticulum, whereas in osmium-fixed A. proteus it is filamentous.⁽⁴⁾ The reason for this difference is unknown, and it is not yet clear whether RuO_4 damages the fibers more than OsO_4 , thus giving the picture of a reticulum, or whether OsO_4 affects the outer fringe to produce fibers or filaments that do not actually exist in living amoebae.

Figure 70. Electron micrograph of *Amoeba proteus* pseudopod fixed in RuO₄ solution. PG, plasmagel; PS, plasmagel; PM, plasmalemma unit membrane; PL, plasmalemma; OF, outer fringe. Epon-812 was used for embedding.

We acknowledge the skilled assistance of Mrs. E. Breyer.

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PATTERNS OF SEGREGATION OF STRUCTURAL ELEMENTS DURING CELL DIVISION

Charles F. Ehret, Nancy Savage
and Judy Alblinger

Introduction

In order to characterize certain properties of organelles of the cell surface in Paramecium (1,2) we have devised several new experimental protocols for the study of organelle ontogeny and techniques for ultrastructural autoradiography. The preliminary experiments reported here indicate the validity of these methods. The properties of interest include molecular stability and composition of organelles, generation time of organelles arising from preorganelles, (3,4) patterns of distribution of new organelles within preexisting organelle complexes and organelle systems, (4,5) patterns of segregation of old organelles amongst progeny cells following cell division, and, ultimately, longevity of individual cell organelles.

Materials and Methods

Cells of Paramecium bursaria (California-3, R. W. Siegel) Syngen 1, mt D, without Chlorella, were grown at 26°C in baked lettuce tea inoculated with Aerobacter aerogenes. The fission rate was about 2 divisions per day. Daily isolation cultures were maintained, and continual surveillance of these cultures permitted recognition and reisolation of proters (the anterior fissions) and opisthes (the posterior fissions) at each cell division. A cell of known age (zero to 12 hours) was treated for a specified interval of minutes or hours by transferring it into an isolation chamber containing sterile lettuce tea and one of several labeled compounds (e.g., DL-leucine-4,5- H^3 hydrochloride, at a concentration of 750 μ c per ml in the chamber). After treatment, the cell was transferred into lettuce tea containing bacteria, and its progeny and their lineage were maintained and recorded.

All fissions of either the first fission (f_1 , 2 cells), the second fission (f_2 , 4 cells) or the third fission (f_3 , 8 cells) were then killed, autoradiographed, and observed in the electron microscope (Siemens Elmiskop I). In some cases every cell of a lineage was oriented and sectioned, according to the single-cell procedures described previously. (6) In other cases a given lineage was used entirely for single-cell whole-mount preparations, following the procedures described below. In the latter case a cell was placed in sterile baked lettuce tea and allowed to swim there for a few minutes to free itself of associated bacteria before transfer to a specimen

grid. It was then sucked up into a fine capillary pipette, along with a very little fluid, and transferred onto a carbon-coated electron microscope grid. The cell dried in a matter of minutes, and drying was accompanied by a discharge of trichocysts. In the microscope, the dried cell was surrounded by a halo, representing the limits of the fluid droplet, with the discharged trichocysts scattered within. The cell contents including the trichocysts were exposed to formaldehyde vapor overnight, and nuclear track emulsion was then applied, following the procedures of Caro⁽⁷⁾ and of Minick,⁽⁸⁾ with minor modifications.

Ilford L-4 nuclear track emulsion, diluted 1:3 with distilled water, was placed in a Coplin jar and heated for 15 min in a water bath at 45°C. The now homogeneous liquid emulsion was then chilled in a 1°C ice bath for 10 min, after which time it was only slightly viscous. After 45 min at room temperature (25°C), the emulsion was considerably more viscous, and was usable in this state. All work with the emulsion was done under Kodak A-0 yellow green safelights.

Emulsion was applied to the specimen grid with a wire loop. The loop was either of fixed (8 mm) or of variable diameter (Figure 71). Best results have been obtained with the fixed diameter design made of 31-gauge Kovar wire. A Pyrex grid holder, similar to Minick's⁽⁸⁾ but facilitating inspection with the light microscope, served as a permanent support for the grids throughout all subsequent procedures (Figures 71-73). The base of the grid holder is cut to the dimensions of an ordinary microscope slide. The glass handle, used to lift the holder in and out of photographic solutions, is bent so that the grids may be examined under the light microscope without obstructing rotation of the microscope objectives (Figure 73).

Affixed to the slide are two columns of five glass posts, 4 mm high, to carry the grids. Grids are fastened to the posts with two very small tabs of double-coated masking tape, placed at the edges. The wire loop is dipped into the emulsion, which is judged for quality and applied to the grid. The grid holders are then put into light-tight boxes in a desiccator containing silica gel and left at room temperature.

At the end of exposure, the grids are developed in Kodak Microdol X in a staining dish for 5 min and then dipped in 1% acetic acid stop bath for 30 sec. The grids are fixed for 3 min in Edwal Quick fix and washed by immersion of the grid holder in distilled water for 5 min.

Observations

Sections of single cells are shown in Figures 74-77 and whole mounts of discharged trichocysts of four cells of different lineages are shown in Figures 78-81. The cell in Figures 74-76 was exposed, at

Figure 71
Equipment developed for ultra-structural autoradiographic work. Left to right: grid holder, and loops of fixed and variable diameter for the application of nuclear emulsion.

Figure 72. Application of film of nuclear emulsion to affixed grid, using each type of loop.

Figure 73. Grid holder on the stage of light microscope. Note especially the relatively short distance between objective and stage, and the clearance of objective by bent handle on the grid-holder.

Figure 74. Electron micrograph of *P. bursaria* exposed at 50 minutes of age for one hour in tritiated leucine and fixed in OsO_4 5 hours after the end of the exposure. Note the uniformly labeled macronucleus (ma, lower left) and micronucleus (mi, upper center). Bacteria in the food vacuoles (fv) are unlabeled (lower right). Nucleolus, nu, in lower left of figure.

Figure 75. Same cell as that in Figure 74. Bacteria in the food vacuoles are labeled (lower left and upper center).

Figure 76. Same cell as that in Figure 74. Elements of the surface membranes and of the cytoplasm are labeled. Note labeled pretrichocyst (pt, lower right).

Figure 77. Cell exposed to tritiated leucine for 2 hr during interphase and fixed in OsO_4 3 hr after the end of the exposure. A heavily labeled mature trichocyst (t), synthesized after exposure to leucine, is seen just below the cell surface (lower center).

Figure 78. Detail of vermiform tracks in emulsion overlying whole-mount preparation of trichocysts. At lower right, a trichocyst tip (t); at upper left the 550 Å spacings on the shaft.

Figure 79. Whole-mount specimen (f0) exhibiting heavily labeled trichocysts.

Figure 80. Whole-mount specimen (f1) exhibiting a persistent heavily-labeled trichocyst and two lightly-labeled ones.

Figure 81. Whole-mount specimen (f3) showing persistent heavily-labeled trichocyst accompanied by other unlabeled, or very lightly labeled trichocysts.

50 minutes of age, to tritiated leucine for 1 hr and was fixed in OsO_4 5 hr after the end of treatment. The overlying nuclear emulsion was exposed for 23 days before developing. At first glance, the distribution of vermiform grains appears uniform and random; closer inspection reveals that this is not the case, even so soon after labeling. Macronucleus, micro-nucleus and cytoplasmic particles are labeled, but the bacteria in some food vacuoles are not (Figure 74); whereas other bacteria in other food vacuoles in the same cell are labeled (Figure 75). Elements of the surface membranes and of the cytoplasm are labeled in Figure 76, including a pretrichocyst at the lower right.

The cell used for Figure 77 was exposed to tritiated leucine for 2 hr during interphase and was fixed after an interval of 3 hr as a late pre-divider. Note the heavily labeled mature trichocyst just below the cell surface in Figure 77. This trichocyst was undoubtedly less than 3 hr old.

Figure 78 shows the 550 Å bending patterns characteristic of the shaft of the discharged trichocyst; superimposed upon the shaft, the vermiform grains indicate sites of deposition of leucine into trichocyst protein during trichocyst formation. Figure 79 is a survey picture of a field of trichocysts from a cell that had been grown for several days in tritiated leucine. The shafts are uniformly and heavily labeled. Considering this cell as f_0 , then by transferring similar young cells to unlabeled lettuce medium and retaining their progeny for whole-mount survey, the results were as follows: at f_1 , each cell yielded heavily labeled trichocysts, as well as many lightly labeled ones (Figure 80); at f_3 , most cells yielded only lightly labeled or unlabeled trichocysts, but a few (Figure 81) contained trichocysts still as heavily labeled as those at f_0 .

Discussion

The transition from pretrichocyst to trichocyst has been calculated from other data⁽⁴⁾ to occur in less than half an hour. In theory, once formed, these organelles remain as structurally stable components of the cell surface, even through cell division;^(4,9) at division, new cortical components including new trichocysts are added to the old ones in a manner topologically analogous to the addition of new wings to an old bisected building.⁽⁹⁾ In its simplest, somewhat unrealistic conception, the consequence of this sort of cell-unit multiplication, carried through three fissions, is shown diagrammatically in Figure 82. At f_1 , most of the surface organelles of the anterior end of the proter (p) and its gullet are old, whereas most of the surface organelles of the posterior end are new. Also at f_1 , most of the surface organelles of the anterior end of the opisthe (o) and its gullet are new, whereas most of the surface organelles

of the posterior end are old. Old gullet and old anterior end are only found in f_1 at p, in f_2 at pp, and in f_3 at ppp. Old posterior end is found only in f_1 at o, in f_2 at oo and in f_3 at ooo. The patterns found thus far with trichocysts on whole mounts (Figures 79-81) are generally in accord with this diagram, although a certain amount of random new element interdigitation (or, better, intercorpularization; see Figure 48 of Reference 4) undoubtedly occurs and remains to be assessed by work in progress.

Figure 82. Lineage diagram (purely schematic, see text) showing proter-opisthe (p-o) genealogies interrelating three fission-generations. Dotted lines indicate metabolically stable pellicular and gullet structures derived from f_0 . Solid lines represent more recently synthesized structures. The cell between f_0 and f_1 is a late pre-divider showing the principal regions of origin of newly synthesized gullet anlage (three bold lines) and pellicular organelles.

Analysis of the patterns of isotope segregation among the fissions of labeled cells has already proved its power in classical studies on modes of DNA multiplication: in bacteria, (10) as well as in cells with conventional chromosomes, (11) semiconservative replication of DNA appears to be the rule. Labeled chromosomes also show semiconservative patterns of segregation of label among their progeny. (12) It is amusing to note that for a single label of the cytoplasmic structural proteins, such as tritiated leucine, a number of apparently different and indeed contradictory patterns can be discerned, depending upon one's point of view. At the

population level, after removal from isotope the cells should be about half as heavily labeled at f_1 as at f_0 , one-quarter as heavily-labeled at f_2 , and about one-eighth as heavily-labeled at f_3 - and, in a gross sense, multiplication of the structural elements of the cell surface viewed from the population level would appear to be dispersive. At the cellular level (and considering only the pellicle system) half of the starting material is retained, the other half is new material (Figure 82): multiplication of the structural elements of the cell surface viewed from the cellular level appears to be semiconservative. But at the organellar level, a trichocyst once labeled appears to retain all its label indefinitely, despite trichocyst multiplication (increase in number) going on in the cell: multiplication of the structural elements of the cell surface viewed from the organellar level appears to be conservative. In the interesting case of the gullet system (Figure 82) multiplication of the structural elements appears in the conservative category whether viewed from cellular or organellar levels.

Had it not been already obvious it should be now, from the examples just given, that segregation patterns per se are insufficient evidence on which to base models for multiplication mechanisms. Thus, semiconservative segregation in chromosomal duplication is not inconsistent with, but neither is it proof of, semiconservative replication of DNA (see the comments of Prescott(13) on this subject), whereas the Meselson and Stahl(10) and Sueoka(11) evidence clearly is. A minimum requirement appears to be that relevant correlation exist between the pattern and its physical basis, as assayed: as a rule, the elements of the pattern must be at the same level of organization as the indicator-parameters of the assay.

In the above paragraphs we have of course departed severely from the particular, already classical, usage of the terms dispersive, semiconservative, and conservative, coined specifically to distinguish between modes of multiplication, and especially of replication, of DNA. If however our intent has succeeded, we have made clear the mathematical similarities that exist and the apparent paradoxes that arise from considerations of any structural patterns at different levels of organization, and particularly for those extra-genic ones that show extraordinarily high degrees of metabolic stability.

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PRECISION TRIMMER OF SPECIMEN BLOCKS FOR ULTRAMICROTOMY

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In order to facilitate the trimming of specimen blocks for ultrathin sectioning, which, in the past, has been done freehand, a precision trimmer has been designed and built (Figure 83). The specimen, embedded in the usual capsular form, is secured in a holder in a central well. A wedge, which tapers toward the capsule, serves as a support for the cutting tool

Figure 83. Precision trimmer for specimen blocks. The diameter of the trimmer is 4 inches (5 inches including the scale) and the height (without wedge) is about 3 inches.

and determines the angle of the sides of the block. The inclined plane of the wedge is fixed at 30° ; this provides for a broad, sturdy base on a pyramidal block. The wedge and an underlying disc, marked off in degrees, can be rotated together about the specimen capsule and locked at any position. Exactly parallel opposing sides can thus be formed on the block face by 180° rotation. The wedge can also be moved, independently, back and forth (radially), permitting variation in the site and size of the trimmed portion. In addition, the capsule holder height is adjusted by a micrometer screw calibrated in $2\text{-}\mu$ steps. When the sliding wedge is moved out of the way, the face of the block can be trimmed by guiding the cutting tool along the horizontal surface of the instrument. Thus, with proper adjustment in three dimensions, precise, neat pyramidal blocks can be fashioned. In trimming the surface, thick sections can be obtained for light microscopy.

Several cutting tools have been tried. The most successful consists of a single-edge razor blade inserted in a specially adapted holder.

At the suggestion of Drs. Daniels and Ehret, who are interested in trimming blocks of single protozoa or other cells, an illuminating device was included. A right-angle surface mirrored prism transmits light through the long axis of the capsule to aid in locating and selecting the specimen.

FURTHER STUDIES ON ACTION SPECTRA AND QUANTUM REQUIREMENTS FOR TRIPHOSPHOPYRIDINE NUCLEOTIDE REDUCTION AND THE FORMATION OF ADENOSINE TRIPHOSPHATE BY SPINACH CHLOROPLASTS

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With the demonstration that isolated and illuminated chloroplasts were able to evolve oxygen, reduce triphosphopyridine nucleotide and phosphorylate adenosine diphosphate,(1,2) it became possible to measure the action spectra and quantum requirements for these processes. In an earlier report of this series(3) we described measurements on the action spectra and quantum efficiency spectra for both reduced triphosphopyridine nucleotide (TPNH) and adenosine triphosphate (ATP) formation. The present experiments extend the earlier data and clarify some of the apparently anomalous results previously obtained. The nonlinear response of ATP formation to increasing intensity of illumination, which was previously reported(4) for white light, has now been found also with monochromatic light. In contrast, the rate of TPNH formation is linear with increasing intensities of either white light or monochromatic light. It is suggested that this difference in kinetics may be caused, at least in part, by adenosine triphosphatase activity.

Results

Effect of Light Intensity. Figure 84, A and B, shows the rate of formation of TPNH and ATP with increasing irradiances at 575 m μ or 675 m μ . The curves resemble those obtained in white light(4) in that the rate of TPNH formation is nearly linear with increasing light intensity, whereas ATP formation shows a small but definite lag at low levels of illumination. The lag in ATP formation was observed only at the lowest levels of absorbed energy but was always found. These effects are reflected in the molar ratios of ATP to TPNH, which approach unity only at the higher levels of irradiance (Figure 84, C and D). Curves of the type illustrated in Figure 84 were constructed at 25 wave lengths from 325 m μ to 750 m μ . Single determinations were also made at additional wave length loci. These data were used to derive the action and quantum efficiency spectra.

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Figure 84. Effects of irradiance at 675 m μ and 575 m μ on the rates of formation of TPNH ($\bullet$ - - $\bullet$) and ATP ($\circ$ - - $\circ$), (A and B), and on the molar ratio of ATP to TPNH ($\circ$ - - $\circ$), (C and D). The maximum incident intensities were 320 and 495 ergs sec⁻¹ mm⁻² at 675 m μ and 575 m μ respectively. Irradiances are expressed as mole-quantum absorbed, or einsteins.

Action Spectrum for TPN Reduction. The action spectrum for TPNH formation is shown in Figure 85. Points on the spectrum were obtained at incident energies in the range giving linear response to increasing light intensity. The spectrum shows a broad peak from 425 to 475 $m\mu$ and a sharp peak at 680 $m\mu$. The red end of the spectrum shows a steep fall at about 695 $m\mu$, but there is only a slow falling off in the blue region. The red peak has a well-defined shoulder at about 600 $m\mu$.

Quantum Requirement for TPN Reduction. The quantum efficiency spectrum of TPNH formation (Figure 86) is essentially linear from about 380 to 685 $m\mu$. There is a slow rise in the quantum requirement below 380 $m\mu$ and a very rapid rise above 685 $m\mu$. The lowest requirement observed was 5.1 quanta per molecule of TPNH at 685 $m\mu$. The average requirement at 430 $m\mu$ is about 1.4 times the average requirement at 685 $m\mu$.

Figure 85

Action spectrum for TPN reduction. The yields were obtained as micro-moles of TPNH formed per incident quantum. The results from each experiment (indicated by the various symbols) were multiplied by an appropriate factor to give the relative yield at 675 $m\mu$ as 100.

Figure 86

Quantum requirement spectrum for TPNH formation. The symbols represent different experiments.

Quantum Requirement for ATP Formation. The calculation of the experimental quantum yield of ATP formation is complicated by the nature of the light intensity curves for ATP formation (Figure 84). The minimal quantum requirement observed at each wave length was calculated from dose-response curves of the type shown in Figure 84. The results are shown in Figure 87. This graph gives the minimal quantum requirement observed at any wave length, regardless of the yield or light intensity at which it was obtained. This quantum efficiency spectrum closely resembles that for TPN reduction (Figure 86). It is essentially linear from about 400 to 685 m μ and shows a sharp "red drop" at about 695 m μ . The lowest requirement observed was 7.1 quanta per molecule of ATP at 685 m μ .

Action Spectrum for TPNH and ATP Formation at Saturating Light Intensities. The action spectrum for the rate of formation of TPNH and ATP at saturating levels of incident energy is shown in Figure 88.

Figure 87
Quantum requirement spectrum for ATP formation.

Figure 88
Action spectrum for TPNH (○) and ATP (●) formation at saturating light intensities.

The spectrum shows a considerable degree of structure, with peaks from 400 to 500 m μ and from 650 to 685 m μ , and rapid drops in response outside this spectral range.

Adenosine Triphosphatase Activity in Chloroplast Fragments. Fragmented spinach chloroplasts exhibited adenosine triphosphatase activity in the dark. About 3 to 6 μ moles of ATP were hydrolyzed per mg of chlorophyll per hour after sonic treatment of the chloroplast fragments.

Discussion

The low adenosine triphosphatase activity which we have observed in the dark is probably identical with that reported by Baltscheffsky.⁽⁵⁾ Its relation to the light-dependent adenosine triphosphatase described by Petrack and Lipmann⁽⁶⁾ and by Avron⁽⁷⁾ is obscure. The properties of the enzyme found in the present experiments have not been examined in any detail; for example, it is not known what effect light may have on the reaction. Furthermore, the adenosine triphosphatase was studied in a reaction mixture markedly different from that in which the photochemical formation of TPNH and ATP was measured. Nevertheless, it seems likely that this enzyme can account, at least in part, for the nature of the light intensity curve for ATP formation previously observed in white light⁽⁴⁾ and now found also in monochromatic light. It is suggested that the actual ratio of ATP to TPNH is 1 at all light intensities, but this may be obscured by the adenosine triphosphatase activity. Thus, at low levels of illumination, the rate of ATP formation is low and a large fraction of the ATP is quickly hydrolyzed. At high levels of light absorption, however, the rate of ATP formation is about 100 μ moles per mg of chlorophyll per hour (Figure 84). This far exceeds the rate of ATP hydrolysis, and the ATP to TPNH ratio thus approaches unity.

The action spectrum for TPNH formation (Figure 85) resembles the partial action spectrum reported by San Pietro et al.⁽⁸⁾ It is also similar to the action spectrum for photophosphorylation in the presence of N-methylphenazonium methosulfate reported by Jagendorf et al.⁽⁹⁾ The close resemblance of the action spectrum (Figure 86) to the absorption spectrum⁽¹⁰⁾ indicates that all the pigments contributing to the absorption spectrum of the chloroplast fragments are able to utilize light to drive the electron transport system leading to TPN reduction. It is not possible to compare our results with the quite different action spectra reported by Kok and Hoch⁽¹¹⁾ for N-methylphenazonium methosulfate photophosphorylation or by Arnon et al.⁽¹²⁾ for TPN reduction and phosphorylation in the presence of vitamin K₃; those workers used very dense suspensions, which are not suitable for the measurement of action spectra.

The relative uniformity in quantum efficiency between 400 and 680 m μ for both TPNH and ATP formation is in contrast with results

described elsewhere.(3,13) A reported rise in quantum requirement in the green portion of the spectrum(3) was based on energy values that were not corrected for scatter. Lumry, Wayrynen, and Spikes(13) also reported a higher quantum requirement in the green for the ferricyanide Hill reaction by Swiss chard chloroplasts. They corrected these values in a later paper(14) and furthermore showed that the ratio of the quantum requirement at 458 $m\mu$ to the requirement at 675 $m\mu$ was about 1.3. A ratio of 1.4 was found in the present experiments. The minimal requirement that we observed was 5.1 quanta per molecule of TPNH and about 7.1 quanta per molecule of ATP at 685 $m\mu$. These values for TPNH and ATP formation are substantially in agreement with those of other workers(15,16) and may be compared with the value of 4 postulated by most two-light theories of photosynthesis.(17)

The quantum requirements for ATP formation (Figure 87) were calculated as the minimal requirement at each wave length. An attempt was also made to calculate the requirements for a constant yield of ATP. Calculations were made for several levels of ATP formation above the lag (about 15 μ moles of ATP per mg of chlorophyll per hour). This method of calculation was unsuccessful outside the limits of maximal chlorophyll absorption because of the nature of the action spectrum for ATP formation at saturating light intensities (Figure 88). Thus, above about 685 $m\mu$, yields were lower than 0.1 μ mole, even at very high light intensity. The method of calculation used to give the data shown in Figure 87 was, therefore, considered to be of greater value. It may be noted that these numbers represent experimentally determined quantum efficiencies. They are not extrapolations of sigmoid curves of the type used by French(18) and criticized by Rabinowitch.(19)

The action spectra at saturating light intensities for both ATP and TPNH formation resemble those obtained by McLeod(20) for oxygen evolution by three species of algae. The bimodality exhibited by these spectra is surprising, since it was formerly thought that the rate of photosynthesis at saturating light intensities would be independent of wave length and would depend on some factor such as limiting substrate or a rate-limiting enzyme.(21) The significance of the structure displayed by these spectra is not clear, but French(22) has suggested that the wavelength dependency of the light-saturated rate of photosynthesis and the Emerson enhancement effect may have a common origin.

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