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RADIATION SURVIVAL IN MAMMALIAN CELLS IN CULTURE AS A FUNCTION OF AGE IN THE GENERATION CYCLE

Warren K. Sinclair

PURPOSE AND METHODS

Earlier reports (for published references, see the 1966 Annual Report⁽¹⁾) have established that survival after X irradiation varies according to the age of the cell within its generation cycle at the time of irradiation. The ultimate purpose of this investigation is to establish the magnitude of this variation as completely as possible and to relate the changes in survival to biochemical processes within the cell (see next report).

It is also intended to relate responses in Chinese hamster cells to those observed by others in other cell lines and to generalize response relationships where this is possible.

The method employs a synchronized population of Chinese hamster cells which normally grow in log phase with a generation time of about 10 hr ($G_1 = 1.5$ hr, $S = 6.0$ hr, $G_2 = 1.5$ hr, $M \sim 1$ hr). Immediately after harvest, cells are nominally at zero age, and responses may be followed thereafter for one or more cycles with a gradually decreasing degree of synchrony. The criterion of effect is usually the reproductive integrity of single cells scored by the number of colonies formed after a suitable incubation period. Irradiation is conducted mainly with 250 kVp X rays, half-value layer 0.9 mm Cu, exposure rate 105 R/min, absorbed dose 0.945 rads/R.

PROGRESS REPORT

Work prior to the current year established that for X-ray lethality, mitosis, and perhaps G_2 , or part of it, was the most sensitive phase of the cycle.

The nature of the experiments by which this was determined limited resolution to about 1 hr, the time taken to kill resistant S cells with high-specific-activity tritiated thymidine, so that survival data are an average for cells within this 1-hr period. Substantial variations could occur within this hour, and a sensitive phase would be most difficult to detect.⁽²⁾ Evidence that a period may exist during which cells are more sensitive than the average for mitotic cells was obtained in the following way.

Plates to be harvested for synchrony were irradiated

with 380 rads of 250 kVp X rays. At intervals of time thereafter, starting with zero (as normally employed for mitotic experiments), plates were harvested by shaking, so that the population selected differed in age at the time of irradiation. The result is shown in Figure 1. Survival varied (A) in a manner which cannot be accounted for by changes in multiplicity or yield (B). Survival reached a minimum (decreased by about a factor of 2 after 380 rads) when the interval between irradiation and shaking was 20 min. Although the exact nature of the population harvested at this time cannot be precisely described, an additional experiment using Colcemid to block cells at metaphase indicated that the sensitive phase is probably after metaphase. Thus, it seems very likely that a sensitive substage of mitosis does exist, but its exact characterization by population methods may be difficult.⁽²⁾

Results of age-response studies in different mammalian cell lines are not easy to compare, mainly because of difference in the lengths of generation cycles and their subdivisions. A review of the published data for a number of cell lines was recently undertaken,⁽³⁾ however, and the following general statements appear to hold. In cell lines with a short G_1 period, cells are most sensitive to lethal damage in mitosis and G_2 , less sensitive in G_1 and least sensitive in the latter part of the S period. In cell lines with a long G_1 , the response is similar, except that there is an additional resistant phase early in G_1 , followed by a sensitive phase near its end. Exceptions appear to occur in some, not all, sublines of L cells in which the second resistant phase does not occur until G_2 . In these cases, however, the changes may be related to biochemical events unrelated to DNA synthesis and, therefore, do not necessarily occur at the same point in the cycle, as these cycles are presently described.⁽⁴⁾

Division delay exhibits a pattern of response which is similar in most cell lines. Delay is considerable for cells in mitosis, small for cells in G_1 ; it increases to a maximum for cells in S , and declines again in G_2 . L cells and human kidney cells may have a longer delay in G_2 than they have in S . The results can be ex-

FIG. 1.—A, Variation of survival after 380 rads of X ray with time between irradiation and shaking for harvested cells. Relative survival is determined by the average number of colonies per inoculated plate divided by yield and cellular

plained in terms of a two-component model of division delay. One component results from the prolongation of the S period, due to the reduced rate of DNA synthesis, and the other, a block in G_2 , is independent of DNA synthesis. The proportion of these two components may vary in different cell lines.⁽³⁾

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multiplicity (cells per colony). B, Variation of yield and multiplicity in the same experiment. (Survival of 1.0 is equal to 0.062 for a yield of 5000 cells/ml, average plating efficiency of 45% and multiplicity of 1.50.)

X-RADIATION SURVIVAL IN MAMMALIAN CELLS AND INHIBITORS OF MACROMOLECULAR SYNTHESIS

Warren K. Sinclair

PURPOSE AND METHODS

The purpose of this study is to relate the variations in X-ray survival occurring during the cell generation cycle to biochemical events occurring within the cell at the time of irradiation.^(1, 2)

The method is to employ Chinese hamster cells in culture synchronized by selection for dividing cells and to examine the X-radiation response of cells inhibited from carrying out various macromolecular syntheses at different stages of the cell cycle. Hydroxyurea (toxic to S cells) and excess thymidine (TdR) (non-toxic to S cells) have been used to inhibit DNA synthesis, and actinomycin D and cycloheximide have been used to inhibit RNA synthesis and protein synthesis, respectively.

PROGRESS REPORT

Preventing the onset of DNA synthesis alone with

either hydroxyurea or excess TdR, by adding these agents to cells in G_1 , causes first an initial sensitization of the lethal response (Figure 2, curve C), compared with the normal X-ray response (curve B), but at about 5 hr the survival increases and rises rapidly to at least the initial value for G_1 cells. Arresting protein synthesis as well in G_1 does not cause sensitization,⁽¹⁾ nor does it if cycloheximide is added during S (curve E). If cycloheximide is added at 5 hr to cells already inhibited with hydroxyurea (curve D), the rise in survival is not affected. A similar pattern is observed if actinomycin D is added to the cells of curve C at 5 hr.

These results indicate that although optimum survival (peak of curve B) can only take place if all macromolecular syntheses take place normally, very marked changes in survival (curve C) take place in the absence of DNA synthesis, and these changes are pre-

sumably related to some other process, as yet unidentified.

The addition of these inhibitors after irradiation also has only a modest effect upon survival, indicating that after irradiation cells do not need these syntheses, at least for the first few hours, in order to survive.⁽²⁾

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FIG. 2.—Effect of cycloheximide (CH) added at 5 hr on synchronous Chinese hamster cells given hydroxyurea (HU) at 1 hr. X rays were given where indicated. (Data not corrected for cellular multiplicity but corrected for drug toxicity.) Curve A, 5 μ g/ml CH alone, added at 5 hr; curve B, 710 rads alone; curve C, 1 mM HU added at 1 hr and 710 rads given at different times. Immediately thereafter, the drug was removed; D, 1 mM HU added at 1 hr, 5 μ g/ml CH added at 5 hr, and 710 rads given at different times. Immediately thereafter, the drug was removed; E, 5 μ g/ml CH added at 5 hr and 710 rads given at different times. Immediately thereafter, the drug was removed.

THE COMBINED LETHAL EFFECT OF HYDROXYUREA AND X RAYS ON CHINESE HAMSTER CELLS IN VITRO

Warren K. Sinclair

PURPOSE AND METHODS

Hydroxyurea has a lethal effect on proliferating Chinese hamster cells in the S phase,^(1, 2) whereas X rays are most damaging to these cells at other stages.⁽³⁾ In addition, a sensitizing effect of hydroxyurea on cells later exposed to X rays was noted.⁽²⁾ These factors indicate that an appropriate combination of hydroxyurea and X rays may effectively control the proliferation of Chinese hamster cells *in vitro* and possibly control other cells *in vivo*. If so, the combination may become uniquely useful for tumor therapy. The immediate purpose of the current investigation is to establish the conditions under which this combination is most effective in Chinese hamster cells *in vitro*.

PROGRESS REPORT

When added prior to exposure, hydroxyurea (1 mM) kills S cells more effectively, does not affect G_2 or mitotic cells, and sensitizes inhibited G_1 cells for a period of about 4 hr (see Figure 2 of previous report,

curve C). Hydroxyurea has no effect when present only during irradiation, but has a further sensitizing effect if present after irradiation.⁽⁴⁾ The sensitizing effect, which increases during about 4 hr of exposure to the drug, is more effective if hydroxyurea is added or kept on cells previously sensitized with hydroxyurea prior to irradiation (Figure 3). In Figure 3, curve D represents the survival of G_1 cells held in hydroxyurea for 4 hr prior to exposure and maintained in the agent for various times thereafter.

It is thus possible to predict the effect of a combination of hydroxyurea and X rays on asynchronous cells. The prediction is borne out experimentally by the data of Figure 4.⁽³⁾ The most effective combination is to add hydroxyurea (1 mM) for 4 hr first, (this kills all S cells in the population and sensitizes the inhibited G_1 cells into which the G_2 and mitotic cells progress), irradiate, and leave hydroxyurea on for at least 4 hr (this causes a further reduction in survival). In Figure 4, curve IV shows that this treatment is

FIG. 3.—Effect of hydroxyurea (HU) (1 mM) added to G_1 cells and present both before and after 710 rads of X radiation. A, Normal response of untreated synchronous cells to 710 rads X radiation; B, survival of synchronous cells to which 1 mM HU was added at 1 hr, following 710 rads of X radiation (drug removed immediately after); compare with Chart 2, curve C, reference 4. For curves C, D, and E irradiation was given after 1 hr of exposure to HU, 4 hr of exposure to HU, and 7 hr of exposure to HU, respectively, and HU kept on afterwards and removed at different times.

much more effective than X rays alone (curve I) or hydroxyurea alone (reduction to ~ 0.3). A modest dose of 300 rads of X rays would cause a survival of 0.65 without hydroxyurea and only 0.03 when hydroxyurea is used as above. Although these results may not be directly applicable *in vivo*, controlled experiments in appropriate *in vivo* tumors appear to be warranted.

FIG. 4.—Effect of 1 mM hydroxyurea upon the X-ray response of asynchronous Chinese hamster cells (attached 2 hr). I, X rays at 2 hr only; II, X rays at 2 hr, HU added for 4 hr; III, HU added at 2 hr, X rays at 6 hr, HU removed immediately; IV, HU added at 2 hr, X rays at 6 hr, HU removed after further 4 hr.

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X-RAY SENSITIVITY OF SYNCHRONIZED CHINESE HAMSTER CELLS IRRADIATED DURING HYPOXIA

Jack Kruw and Warren K. Sinclair

PURPOSE AND METHODS

The purpose of this investigation is to determine if the variation in X-ray sensitivity evident as the cell progresses through its cycle⁽¹⁻³⁾ is differentially affected by its state of oxygenation at the time of irradiation. It is well known that well oxygenated cells are more sensitive to X radiation than hypoxic cells.⁽⁴⁾

The method employs a synchronized population of Chinese hamster cells, which normally grow in log phase with a generation time of about 10 hr ($G_1 = 1.5$ hr, $S = 6$ hr, $G_2 = 1.5$ hr, $M \sim 1$ hr). Synchronized cells obtained by mitotic selection are inoculated onto plastic Petri dishes and followed throughout subsequent generation cycles.⁽¹⁻³⁾

The Petri dishes, with the medium removed and lids on, were placed in a Perspex box with a volume of 1.7 liters and were deoxygenated by passing humidified 98% N_2 and 2% CO_2 (measured to have less than 20 ppm O_2) through the box at the rate of 2 liter/min for 30 min. The controls were treated in a similar manner with air and 2% CO_2 . The valves of the boxes were closed immediately after the flush and were reopened after irradiation. X-ray exposures were conducted with a 250-kVp X-ray machine, half-value layer 0.9 mm copper, exposure rate about 160 R/min (0.945 rad/R) at the exposure level of the cells.

The criterion of effect was the reproductive integrity of individual cells, irradiated mainly as two-cell microcolonies,⁽¹⁾ scored by the number of colonies formed after a suitable incubation period (8–10 days).

FIG. 5.—A, Grains per 100 cells counted after pulse-labeling with 3H -thymidine ($0.3 \mu Ci/ml$, $3 Ci/mmol$, 15 min) at different times during the cell cycle; B, fraction of colonies surviving after irradiation of cells in air with 750 R; C, fraction of colonies surviving after irradiation of cells in nitrogen with 1725 R.

FIG. 6.—Survival curves for cells irradiated either in air or nitrogen when in G_1 (1.5 hr), early S (3.5 hr), and G_2 (9.5 hr). The apparently high survival and resistant-tail curve in G_2 is due to contaminating S cells.⁽³⁾

FIG. 7.—Survival curves for cells irradiated either in air or nitrogen in middle S (5.0 hr), and late S (7.0 hr).

PROGRESS REPORT

With the above apparatus and using cells of subline V79-285B, an OER (oxygen enhancement ratio) of 2 to 2.3 was obtained when asynchronous cell cultures were irradiated.

When synchronized cells were removed at intervals from the incubator and irradiated in air (750 R) or N₂ (1725 R), assuming an OER of 2.3, the resulting curves of survival against cell age were very similar (Figure 5), indicating no substantial variation of OER with age.

X-ray survival curves for cells in G₁ (1.5 hr), early S (3.5 hr), middle S (5.0 hr), late S (7.0 hr), and G₂ (9.5 hr) are shown in Figures 6 and 7. The air and nitrogen survival curves are similar in shape at each point except for the dose modifying factor. If, using the data from Figures 6 and 7, one plots survival after 800 R in air and 1600 R in N₂ versus time after synchronization, the resulting curves are almost identical.

Upon repeating the experiment with a different subline (X325) of small-colony cells⁽⁵⁾ having a longer G₁ and generation time, the results were essentially

the same. In this experiment a lower OER (~1.7) was obtained, but approximately the same OER was observed throughout the whole cycle.

CONCLUSION

The variation in radiosensitivity as the cell progresses through its cycle must be due to factors other than change in oxygen tension within the cell.

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EFFECTS OF TEMPERATURE ON MACROMOLECULAR SYNTHESIS IN MAMMALIAN CELLS

Jack Kruw and Warren K. Sinclair

PURPOSE AND METHODS

There are both practical and fundamental reasons for examining the rate at which mammalian cells synthesize macromolecules at different temperatures. Practically, in tissue culture work *in vitro*, cells grown normally at 37°C are often exposed to other suboptimal temperatures (e.g., room temperature ~25°C, or 4°C) for certain operations. When such temperature changes occur, does the synthesis of macromolecules alter in the same way or are differential changes incurred which may alter the subsequent behavior or response of cells to other agents?

To investigate these points, synchronized Chinese hamster⁽¹⁾ cells inoculated onto plastic Petri dishes (52 mm in diameter) containing a total of 6 ml of medium were incubated for 6 hr, at which time the cells had reached the middle of the S period and were synthesizing DNA at about the maximum rate.⁽²⁾ At this time protein and RNA synthesis were also proceeding at about the maximum rate.⁽³⁾ Pulse-labeling with ³H-thymidine (0.2 ml of 10 μCi/ml), ³H-uridine (0.2 ml, 16.6 μCi/ml), or ³H-leucine (0.2 ml, 200 μCi/ml, each at about 4 Ci/mM), was conducted at various times

after the removal of dishes from a 37°C incubator in which the pH of the medium was maintained at about 7.3. Cells were later fixed, liquid emulsion was added, and the dishes developed and stained after 7 days' exposure. Grains per 50 cells were scored in the microscope. Temperature was measured by means of a thermistor inserted into the medium through a hole in the lid of the dish, and pH was measured through another hole using a Beckman Model 76 pH-meter.

PROGRESS REPORT

Figure 8 shows the variation in temperature and pH, when dishes are removed from a 37°C incubator (zero time) into a room which had a temperature of 26°C. It takes about 50 min for the 6 ml of medium and cells to equilibrate to room temperature. The change in pH is due to loss of carbon dioxide from the medium.

Figure 9 shows the variation in ³H-thymidine uptake during the same experiment. After 1 hr, although temperature equilibration has been achieved (Figure 8), cells were still taking up ³H-thymidine at a decreasing rate.

FIG. 8.—Variation in temperature and pH with time of 6 ml of medium on a 52-mm tissue culture dish after removal from a 37°C environment to one at 26°C.

FIG. 10.—Variation in percentage of labeled cells with temperature and time. A cell was considered labeled if it took up 1 grain in a 5-min pulse-labeling period.

FIG. 9.—Variation in ³H-thymidine uptake with temperature and time. The width of the bars indicates the length of the pulse-labeling period at 26°C. A cell was considered labeled if it took up 3 grains in a 15-min pulse-labeling period. All grain counts were normalized to a 15-min labeling period.

FIG. 11.—Variation in ³H-thymidine, ³H-uridine, and ³H-leucine uptake with temperature and time. A cell was considered labeled if it took up 1 grain in a 5-min pulse-labeling period.

To investigate this further, dishes of synchronized cells incubated at 37°C for 6 hr were transferred to environmental rooms controlled at 23°C, 21°C, and 3°C. Between 45 and 60 min, the grain counts were 0.1, 3.4, 4.5, and 5.2 grains/cell/15 min at 3°, 21°, 23°, and 26°C, respectively. Between 75 and 90 min, these rates had fallen to 0.06 (equal to background) at 3°C and 1.4 grains/cell/15 min at 21°C. Thus cells continue to take up ³H-thymidine slowly even after temperature equilibration has occurred.

In a further experiment, 6-hr cells at 37°C were transferred to 3°C for 15 min, during which the temperature of the medium fell to 5.4°C. Cells were then transferred to 26°C and pulse-labeled for 5 min at intervals of time thereafter with ³H-thymidine, ³H-uridine, or ³H-leucine. The results are shown in Figures 10 and 11, zero time corresponding to the time of transfer from 3°C and 26°C. Cells with more than 1 grain/cell/15 min were considered labeled since the background during this interval was such that less than 4% of cells on control plates had a single grain.

Figure 10 shows that even at 20°C, almost 100% of

the cells were synthesizing protein, while 60% were synthesizing RNA and only about 30% were synthesizing DNA, although at the time of removal from 37°C, practically 100% of the cells were synthesizing DNA. Together with Figure 11, this indicates that protein synthesis is either not arrested at 5.4°C as effectively as RNA and DNA synthesis, or that it begins again more rapidly when the temperature rises. DNA synthesis is the most affected by the temperature change, RNA synthesis less so and protein synthesis least of all.

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MITOTIC DELAY AND CHROMOSOMAL ABERRATIONS INDUCED BY X RAYS IN SYNCHRONIZED CHINESE HAMSTER CELLS IN VITRO

Chi K. Yu and Warren K. Sinclair

FIG. 12.—Mitotic delay vs. exposure for the least sensitive stage (G_1 , 1 hr) and most sensitive stage (middle to late S , 6-7 hr) of the generation cycle. The former has a slope of 0.3 hr/100 rads (0.945 rads/R) and latter a slope of 0.7 hr/100 rads.

PURPOSE AND METHODS

Preliminary studies of mitotic delay and chromosomal aberrations induced by X irradiation were presented in 1966 Annual Report.⁽¹⁾ A synchronized Chinese hamster (*Cricetulus griseus*) cell strain V79-285B was exposed to X radiation at various stages of the cell cycle, namely, G_1 , early S , late S , G_2 , and M . Exposures of 250 R, 500 R, and 750 R were used for mitotic delay studies and 500 R and 750 R for chromosomal aberration studies. The data obtained from these experiments have been analyzed and will be published shortly.⁽²⁾

PROGRESS REPORT

It was found that the mitotic delay varied according to the stage of the cell cycle at the time of irradiation. The delay of mitosis increased approximately linearly with increasing exposure or absorbed dose (Figure 12), with a minimum value of about 0.3 hr/100 rads for cells irradiated in G_1 (at 1 hr) to a maximum value of 0.7 hr/100 rads for cells irradiated in middle to late S . Cells irradiated at other times have also a near linear response with an intermediate value of slope.

For chromosomal aberration studies, the number of aberrant cells after both exposures was highest when cells were irradiated in G_2 and early S . Cells irradi-

FIG. 13.—Chromosomal segments (pairs) induced at G_1 (1 hr after synchronization) after an exposure of 750 R.

ated during S or G_2 produced mostly chromatid aberrations (i.e., chromatid lesions and exchanges), while cells irradiated in mitosis or G_1 exhibited principally chromosome aberrations. Dicentric chromosomes occurred only at relatively low frequency. In some cases, chromosome aberrations (e.g., dicentric chromosomes) and chromatid aberrations (e.g., chromatid exchange) occurred in the same cell, especially in late G_1 and early S , presumably as a result of the asynchronous

duplication of chromosomes (already known from labeling studies in Chinese hamster and other cells^(3, 4)).

One further feature should be added. A number of chromosomal segments were found in these cells, especially those irradiated in G_1 . The chromosomal segments usually appeared as pairs (Figure 13) and were both large segments and fragments. Their origin is uncertain, i.e., there is no direct cytological evidence to indicate whether they were derived from an iso-

chromatid lesion or a chromosome break. However, if we plot the frequency of these chromosomal segments as a function of time of irradiation during the cell cycle (Figure 14), we see that the form of the response is similar to that of chromosome breaks. Therefore, it is likely that these acentric segments originated from chromosome breaks rather than chromatid breaks.

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FIG. 14.—Chromosomal aberrations obtained after 750 R from the populations irradiated at different times within the cycle. Crosses and solid line indicate chromatid aberrations. Dots and broken line indicate chromosome aberrations. Circles and dotted line indicate acentric segments.

ULTRAVIOLET SENSITIVITY OF SYNCHRONIZED CHINESE HAMSTER CELLS AT VARIOUS STAGES OF THE CELL CYCLE

Warren K. Sinclair and Antun Han*

PURPOSE AND METHODS

Previous studies⁽¹⁻⁴⁾ have established a marked variation in the lethal response to X rays of Chinese hamster cells as they age between one division and the next. These cells were found to be most sensitive in G_1 , G_2 and mitosis. Initial studies with ultraviolet light (UVL)⁽²⁾ indicated a markedly different survival response with age of these cells from their response to X rays. The purpose of this investigation is to establish the age-response of Chinese hamster cells to UVL more completely, in order to exploit the differences in their age response between X rays and UVL to yield a better understanding of both. Further studies will be concerned with the differences in action of X rays and UVL on mammalian cells with regard to the progression of cells, the repair of sublethal damage, and the interaction between the two radiations.

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The method employs a population of Chinese hamster cells, subline V79-S171, synchronized by selection for dividing cells. The details of the harvesting procedure, as well as the methods of measurement of degree of synchrony, have been described in detail elsewhere.⁽²⁾ The criterion of effect is reproductive integrity of single cells, scored by the number of clones formed after a given incubation period (8-10 days) after irradiation. A General Electric germicidal lamp was used as the source of ultraviolet light. The predominant wavelength from this lamp is 2537 Å. The incident dose, at the irradiation surface level, was 4.3 ergs/mm²/sec, as determined by an Eppley thermopile. Cells were irradiated attached to the dish, and medium was sucked off just prior to irradiation and added again immediately afterward.

PROGRESS REPORT

The response of the cell population to four different

doses of UVL at different times in the cycle is shown in Figure 15. The general pattern is that cells are resistant in G_1 , sensitive as cells move into the S phase, and resistant again in G_2 . At 10 hr, the population comprises a mixture of mainly G_2 cells with some mitotic cells, and the results at this time represent an average survival for both G_2 and mitotic cells.

Survival curves were obtained in two sets of experiments, one set for intermitotic cells and the other for mitotic cells alone, using the method of irradiation just prior to harvest.⁽⁴⁾ The results are shown in Figure 16. Mitotic cells are the most resistant moiety, at least for the first decade—much more resistant than S cells and more resistant than either G_1 or G_2 cells. The values for D_0 between the most sensitive (S) and resistant (M) cells differ by at least a factor of two, even when the extrapolation numbers are about the same (4.0-hr cells, early S , compared with mitotic cells). There is no significant difference in survival between early and late S cells. The same is true for G_1 and G_2 cells, although G_2 cells represent a less pure population. Variations in D_0 are accompanied by some variations in extrapolation number. The data are not sufficiently precise, as yet, to make firm conclusions about the

FIG. 15.—Age-response of Chinese hamster cells to four different doses of ultraviolet light (dose rate = 4.3 ergs/mm²/sec).

FIG. 16.—Single-cell survival data for various stages of the cell cycle (dose rate = 4.3 ergs/mm²/sec).

variations in extrapolation number, as compared with those in D_0 , throughout the cycle.

On the basis of the data presented so far, it can be concluded that the response of synchronized cells to UVL throughout the cycle differs markedly from the response to X rays. In fact, for these cells their lethal response to UVL is almost the inverse of their response to X rays. It should be emphasized that the most remarkable difference in survival of Chinese hamster cells after X rays and UVL is observed in mitotic cells, which are the most sensitive to X rays⁽⁴⁾ and apparently are most resistant to UVL.

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EFFECT OF CYSTEAMINE UPON THE X-RAY RESPONSE OF CHINESE HAMSTER CELLS DURING THE GENERATION CYCLE

Warren K. Sinclair

PURPOSES AND METHODS

Cysteamine protects mammalian systems *in vivo* and *in vitro* against the effects of X radiation (see, for example, references 1 and 2). This protective action may depend upon the concentration of unidentified constituents within the cell, which could vary during the cell generation cycle. A study of the protective action of cysteamine upon cells irradiated at different stages of the cell cycle, therefore, may provide information not only on the action of cysteamine but also on the reasons for the variation of X-ray survival throughout the cycle.^(3, 4)

The method is to use Chinese hamster cells synchronized by mitotic selection and to irradiate them at different times after synchronization in both the presence

FIG. 17.—The survival of synchronous Chinese hamster cells irradiated at different stages during the cell cycle with 710 rads of 250-kVp X rays. Curve A, no cysteamine; B, 5 mM cysteamine present during exposure; C, 50 mM cysteamine present during exposure.

and absence of different levels of cysteamine. 250-kVp X rays, HVL 0.9 mm Cu, at ~100 rads/min were used.

PROGRESS REPORT

The results of an experiment in which different con-

FIG. 18.—Survival of G_1 , 2.5-hr (curve A) and late S, 7-hr (curve B) cells after 710 rads of 250-kVp X rays in different concentrations of cysteamine.

FIG. 19.—Survival curves for G_1 and late S cells with and without cysteamine (50 mM). Curves A (G_1) and B (late S) no cysteamine. Curves C (G_1) and D (late S) with 50 mM cysteamine. Curve E, G_1 cells with 50 mM cysteamine irradiated at higher dose rate (150 rads/min).

centrations of cysteamine were added to synchronous cells just prior to irradiation and removed just afterward are shown in Figure 17. Curve A shows the normal variation in survival after 710 rads of 250-kVp X radiation at various stages of the cell cycle for this subline (V79-S171). Curves B and C show the effect of adding 5 mM or 50 mM cysteamine just prior to irradiation and removing it just afterward. These levels of cysteamine were not toxic to Chinese hamster cells during the 15- to 20-min period required for its addition, irradiation, and removal. The approximate lengths of G_1 , S , and G_2 , as determined by pulse-labeling with ^3H -thymidine are also shown in Figure 17.

The variation in X-ray survival, with concentration of cysteamine for 2.5-hr cells (curve A) and 7-hr cells (curve B) determined by additional experiments like those in Figure 17, is shown in Figure 18. These experiments indicate that 50mM cysteamine affords maximum protection of 7-hr cells, and near maximum for 2.5-hr cells. Evidently, the effect of an adequate cysteamine concentration is to level off the response throughout the cell cycle so that there is very little variation throughout it.

Survival curves for these cells, at different cell ages, were determined both in the presence and absence of 50 mM cysteamine. The results of these experiments are shown in Figure 19. Curves A and B are for G_1 and late S cells, respectively, without cysteamine. Curves C and D are for G_1 and late S cells with 50 mM cysteamine. It is evident that 50 mM cysteamine provides a high degree of protection; the Dose Modifying Factor (DMF), comparing doses at the same survival level during the first decade, is about 2.7 for S cells and 4.2 for G_1 cells. This agrees well with published values of DMF for

asynchronous cells.⁽⁵⁾ Curve E, for G_1 cells, was obtained in a separate experiment with 50 mM cysteamine and higher doses at a dose rate of 150 rads/min.

The most significant feature of these studies is the levelling-off of the X-ray response during the cell cycle as a result of cysteamine protection. This gives a strong indication that at least part of the reason for the variations in X-ray survival normally evident during the cell cycle may be the level of radioprotective constituents which the cell possesses at given stages. Cysteamine supplies the necessary protective constituents in excess, so that no variation in response is observed throughout the cycle. It may be noted that since the age response for survival has the same form for both oxygenated and hypoxic cells,⁽⁶⁾ the action of cysteamine presumably does not depend on the state of oxygenation in the cell.

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ACTION SPECTRUM FOR PROTECTION OF E. COLI AGAINST ULTRAVIOLET EFFECTS BY ATEBRIN

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PURPOSE AND METHODS

Dyes that have been shown to protect against short ultraviolet (UV) effects have an absorption peak in the short UV range.⁽¹⁾ Although shielding has been considered in computing the incident dose to the cells, possible local shielding by dye molecules bound to the DNA may be the cause of this protection. If local shielding is the only factor in protection, the entire phenomenon is of little importance to explanations of the molecular

biology of the cell. If local shielding is a major factor in protection, the action spectrum for cell protection should correspond to the absorption spectrum of the dye in the short UV range.

In this study, the sensitivity of *E. coli* to short ultraviolet radiant energy, as a function of wavelength, was studied in the presence and absence of an acridine dye, atebtrin (quinacrine, mepacrine). Metabolic properties of atebtrin, an antimalarial agent, have been investigated recently.⁽²⁾ Evidence has been presented that atebtrin binds to native DNA through intercalation.⁽³⁾

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FIG. 20.—Ultraviolet (254 nm) dose-inactivation curves of *E. coli* WP2-hcr⁻ in the absence and presence of atebtrin. Irradiance was 7.1 ergs/mm²/sec.

Procedures used in this study have been given elsewhere.⁽¹⁾ Cell suspensions containing approximately 10⁷ cells/ml in 1 mM phosphate buffer were used. Cell suspensions were 2 mm thick to minimize shielding cor-

rections. The suspensions were stirred with a gas jet impinging on the suspension surface. The source of radiant energy was a Bausch and Lomb Hi Intensity monochromator with a 200-watt xenon lamp, a UV-visible grating, and continuously adjustable slits. A low-pressure Hg lamp (Penray), with 90% of its emission at 2537 Å, was used in the dose rate measure-

FIG. 21.—Effect of irradiance (254 nm ultraviolet radiant energy) on the inactivation constant (k) and the shoulder constant (n) of *E. coli* WP2-hcr⁻.

TABLE 1. SUMMARY OF SURVIVAL CURVES OF *E. coli* WP2-hcr⁻ OF THE INACTIVATION CONSTANT (k) AND THE SHOULDER CONSTANT (n)

Wavelength, nm	Half band-width, nm	Irradiance, ergs/mm ² /sec	Atebrin conc., M	$k \pm \text{S.E.}$	$n \pm \text{S.E.}$
254		1.60	—	0.0712 $\pm$ 0.0014	3.15 $\pm$ 0.35
254		3.41	—	0.0719 $\pm$ 0.0018	2.33 $\pm$ 0.32
254		5.89	—	0.0987 $\pm$ 0.0016	2.26 $\pm$ 0.22
254		8.11	—	0.1006 $\pm$ 0.0026	2.31 $\pm$ 0.31
254		11.9	—	0.1132 $\pm$ 0.0018	2.42 $\pm$ 0.22
254		19.1	—	0.1604 $\pm$ 0.0031	3.50 $\pm$ 0.38
254		1.95	5 $\times$ 10 ⁻⁶	0.0429 $\pm$ 0.0013	1.77 $\pm$ 0.25
254		8.58	5 $\times$ 10 ⁻⁶	0.0581 $\pm$ 0.0013	1.60 $\pm$ 0.16
230	21	3.33	—	0.0360 $\pm$ 0.0006	2.84 $\pm$ 0.27
240	16	3.71	—	0.0295 $\pm$ 0.0005	1.95 $\pm$ 0.16
250	13	3.33	—	0.0522 $\pm$ 0.0010	1.72 $\pm$ 0.14
260	11	3.49	—	0.0709 $\pm$ 0.0011	2.49 $\pm$ 0.17
270	8	3.74	—	0.0771 $\pm$ 0.0024	2.43 $\pm$ 0.31
280	6	3.47	—	0.0537 $\pm$ 0.0015	2.40 $\pm$ 0.23
290	6	3.47	—	0.0237 $\pm$ 0.0011	1.37 $\pm$ 0.18
300	6	3.60	—	0.00576 $\pm$ 0.0002	3.59 $\pm$ 0.50
230	21	3.14	5 $\times$ 10 ⁻⁶	0.0123 $\pm$ 0.0004	2.81 $\pm$ 0.41
240	16	3.96	5 $\times$ 10 ⁻⁶	0.0112 $\pm$ 0.0005	1.48 $\pm$ 0.15
250	13	3.44	5 $\times$ 10 ⁻⁶	0.0239 $\pm$ 0.0005	2.46 $\pm$ 0.17
260	11	3.47	5 $\times$ 10 ⁻⁶	0.0376 $\pm$ 0.0015	1.61 $\pm$ 0.27
270	8	3.96	5 $\times$ 10 ⁻⁶	0.0398 $\pm$ 0.0009	1.93 $\pm$ 0.29
280	7	3.58	5 $\times$ 10 ⁻⁶	0.0290 $\pm$ 0.0005	1.88 $\pm$ 0.16
290	6	3.41	5 $\times$ 10 ⁻⁶	0.0132 $\pm$ 0.0004	1.40 $\pm$ 0.15
300	6	3.63	5 $\times$ 10 ⁻⁶	0.00341 $\pm$ 0.00008	3.10 $\pm$ 0.27

ments. The same irradiation vessel was used throughout this study. Dosimetry was done with a Schwarz vacuum thermopile and a Keithley 150 B microvoltammeter standardized against a National Bureau of Standards lamp.

Survival curves were described with the expression

$$S = 1 - (1 - e^{-kD})^n,$$

where S is the surviving fraction, D is dose in ergs/mm², k is the inactivation constant—the slope of the exponential part of the log-transformed dose-survival curve—and n , the “shoulder constant,” is the y -axis intercept of the extrapolation of the exponential part of the curve. All responses were obtained over about three log₁₀ cycles.

Three complete series of experiments were run, each giving essentially the same results. The final run is presented here, in which irradiance was kept within close limits, and multiple irradiance measurements were made before and after each exposure.

PROGRESS REPORT

The basic protection effect in *E. coli* WP2-hcr⁻ shown by atebrin is given in Figure 20. Saturation of the effect is evident at the higher concentrations. A sizable dose-rate effect was observed for 2537 Å inactivation of *E. coli* WP2-hcr⁻ (Figure 21 and Table 1). Sensitivity varied a factor of more than two over the irradiance range of 1.6 ergs/mm²/sec to 19.1 ergs/

FIG. 22.—Effect of wavelength of ultraviolet radiant energy on the inactivation constant (k) in the absence and presence of atebrin. Irradiance was between 3.14 and 3.96 ergs/mm²/sec.

FIG. 23.—Effect of wavelength of radiant energy on the efficiency of protection against inactivation (ratio: k no dye/ k dye) by 5 μ M atebrin.

mm²/sec. The value of n did not show a corresponding systematic change over this range. Because of the pronounced dose-rate effect, we kept the irradiance for all of the action spectrum experiments within $\pm 20\%$ (Table 1).

Action spectrum results for *E. coli* WP2-hcr⁻ in the presence and absence of atebrin (5 μ M) are shown in Table 1 and Figure 22. This low level of atebrin was used to minimize the correction required from physical shielding by the dye. The shoulder constant n showed only random variation over this range (Table 1). The departure of the action spectrum from the absorption spectrum of DNA was repeatable and appears to be significant. Long wavelengths appear to be more efficient than they would be expected to be on the basis of simple absorption by DNA. It is possible that another kind of damage (non-dimer) may be of increasing importance at the longer wavelengths. Further investigation will be required to establish these relationships.

The relationship between the protection ratio and the absorption spectrum of atebrin is shown in Figure 23. There is little correspondence between the absorption of atebrin and protection against UV inactivation. If local shielding by acridine dyes plays a major role in protection, then protection should closely correspond to absorption by the dye. The absence of such a correspondence demonstrates that other mechanisms than local shielding play the major role in protection.

The decrease in protection from short wavelengths to long wavelengths is not understood but may provide

a basis for investigation into the nature of UV-induced alterations in DNA.

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MOLECULAR INTERACTIONS OF BIOLOGICALLY ACTIVE MOLECULES

David J. Blears, Steven S. Danyluk, Kunio Hikichi, and Frank E. Hruska

PURPOSE AND METHODS

The objectives of this investigation have been summarized in a previous report.⁽¹⁾ In the past year most of the effort has been concentrated in the area of nucleotide and oligonucleotide structures and interactions in solution. The emphasis in this direction stems from the possibility of applying high-resolution nuclear magnetic resonance techniques to sequence and structure determinations of nucleic acids.

One of the main problems in sorting out the physical and chemical nature of processes, such as transcription and replication, is the lack of knowledge concerning the

sequences and structures of the interacting molecules. At the present time the sequences of nucleic acids and proteins are determined by very lengthy degradative techniques. It is of considerable importance to find non-destructive methods of speeding up this process, and it is hoped that the results of the present study will contribute in this direction.

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I. A NUCLEAR MAGNETIC RESONANCE STUDY OF NUCLEIC ACID DERIVATIVES IN SOLUTION

A. CONFORMATION AND RING-CHAIN INTERACTIONS OF 5'-NUCLEOTIDES

Frank E. Hruska and Steven S. Danyluk

PURPOSE AND METHODS

The conformation of a nucleotide in solution is of primary importance in fundamental biological processes such as replication and transcription of nucleic acids. For this reason the structures of nucleosides and nucleotides have been intensively studied by a variety of physico-chemical techniques.⁽¹⁻³⁾ One aspect which has received particular attention⁽⁴⁾ is the ring-chain interaction between the base ring and the phosphate group in nucleotides. From simple stereochemical considerations it can be shown that a ring-chain interaction would be particularly favored in purine 5'-nucleotides in the *anti** conformation at low pH's. Under these conditions a strong electrostatic interaction can occur between the negatively-charged phosphate group and the positively-charged purine ring. Such an interaction will

have important consequences upon the conformation and ion-binding properties of the nucleotide. The objective of the present investigation is to obtain further knowledge about ring-chain interactions using the technique of high-resolution NMR.

PROGRESS REPORT

The high-resolution proton magnetic resonance spectra were measured† at 32°C for a wide variety of purine and pyrimidine nucleosides and nucleotides over the pD range 1 to 8.2. A typical set of results is illustrated in Figures 24 and 25, where the pD dependence is shown for the base-ring protons of a number of adenine derivatives. Similar sets of data were obtained for the other compounds studied.

The results can be summarized as follows:

* An *anti* conformation is one for which the torsion angle about the C-N bond lies in the range of -30° .

† The spectra were measured with a Varian DA-60 spectrometer operating in the internal-lock mode.

FIG. 24.—Proton chemical shifts [cps relative to external tetramethylsilane (TMS)] versus pD of the solution. a, Adenine-H₂ (●) and H₈ (○); adenosine-H₂ (▲) and H₈ (△); and adenosine-5'-monophosphate-H₂ (■) and H₈ (□). b, H_{1'} of adenosine (□) and of adenosine-5'-monophosphate (○). c, 2'-Deoxyadenosine-5'-monophosphate-H₂ (■), H₈ (□) and H_{1'} (○).

$pD < 5.5$

Low-field shifts of the base-ring proton signals were noted with decreasing pD for all of the purine derivatives studied. The largest deshieldings were observed for guanine and xanthosine derivatives. These shift changes are attributed to a protonation of the base-rings in all cases.⁽⁵⁾ For the adenine derivatives protonation occurs in the 6-membered ring at the N₁ position, while for the guanine and xanthine derivatives protonation takes place at the N₇ position of the imidazole ring. The protonation reactions are confirmed by the good agreement observed between pK values derived from the chemical shift curves and values obtained from titration measurements.⁽⁶⁾ In contrast with the purine compounds, the pyrimidine bases and nucleo-

sides show a negligible shift change of the base ring protons below pD 5.5, while the 5'-nucleotides show small deshieldings above pD 2.0. The pyrimidine nucleoside results are not unexpected since the base rings do not possess an ionizable group in the pD region covered.

$pD > 5.5$

No shift change is noted for the base-ring protons of the free bases and nucleosides in the pD range 5 to 8.2, indicating the absence of any ionization process in this region. For nucleotides, on the other hand, this pD range covers the region in which secondary phosphate ionization occurs for all of the nucleotides studied. However, the base-ring proton shifts for all of the nucleotides, except the 5'-derivatives, were constant with increasing pD above pD 5.5. For the purine 5'-deriva-

FIG. 25.—Proton chemical shifts (cps relative to external TMS) versus pD of the solution. A, Adenosine-2',3'-cyclic monophosphate- H_2 (●) and H_8 (○); adenosine-3',5'-cyclic monophosphate- H_2 (▲) and H_8 (△); and adenosine-2'- and -3'-monophosphate (mixture)- H_2 (■) and H_8 (□). B, H_1 of compounds in section a; symbols have the same meaning.

tives a significant deshielding was observed for the imidazole ring protons (H_8) but not for the pyrimidine ring protons, i.e., H_2 in adenine-5'-monophosphate.

These deshieldings can be rationalized on the basis of a preferred *anti* conformation for the base and furanosyl groups. It can be shown with Courtault's models that in this conformation, rotation of the phosphate group about the C_5' —O bond in the 5'-nucleotides can bring the phosphate group into close proximity to the H_8 proton. In the β -2' and -3' isomers (and the cyclic isomers) a similar approach is sterically hindered by the furanosyl ring.* Furthermore, in this

* The distance of approach of the 5'-phosphate group to H_8 is approximately the same in the *anti* conformation as the approach of this group to H_2 in the *syn* conformation.

conformation the distance between the phosphate group and H_2 is too great for any effect upon the shift of this proton. Ionization of the 5'-phosphate group can then give rise to a deshielding of the H_8 proton, either by an increase in the electric field effect due to the PO_4^- group or by a hydrogen-bonding interaction between H_8 and the oxygen atoms. Although it is not feasible to assess the relative contributions from these interactions, nevertheless the NMR results confirm the presence of a ring-chain interaction in the pH range 5.0 to 8.2. This is in contrast with the conclusions reached from extensive optical and ionization studies,⁽⁴⁾ which indicate an electrostatic ring-chain interaction below pH $\sim$ 5.0, where the base ring has a positive charge, and no interaction above this pH, where the ring is uncharged. The apparent anomaly arises because the chemical shift is more sensitive to weak local environmental changes than are the other measurements. It can be noted that although the base rings of purines and their derivatives are electrically neutral at pH 5 to 9, the H_8 protons can, nevertheless, form hydrogen bonds with suitable acceptors in both aqueous and nonaqueous media.⁽⁷⁾ This ability is due to the electropositive nature of the C_8 carbon atom and may account for the intramolecular effects observed in the 5'-nucleotides.

It is of interest to note that the shift changes in the pD range 5.0 to 8.2 for adenosine-5'-monophosphate and 2'-deoxyadenosine-5'-monophosphate (and also for the corresponding guanosine derivatives) are quite similar, i.e., 7.5 and 6.0 cps (Figure 24) indicating that any conformational differences due to the presence of a possible hydrogen bond between 2'—OH and N_3 ⁽⁸⁾ are not reflected in the effect of the PO_4^- group upon the ring shift.

For pyrimidine-5'-nucleotides a small deshielding was observed in the pD range 5.5 to 8.2. As in the case of the purine-5'-nucleotides the deshieldings can be attributed to the effects of secondary phosphate ionization.

CONCLUSIONS

The pD dependence of the proton chemical shifts of a number of purine and pyrimidine bases, and their nucleosides and nucleotides, has been studied in D_2O at 32°C. It is concluded from the NMR results that a weak ring-chain interaction of an electrostatic and/or hydrogen-bonding type is operative in both the 5'-purine and -pyrimidine nucleotides over the pD range 1 to 8.2. No interaction of this type is indicated for the isomeric 2'- and 3'-nucleotides. The present results are consistent with a preferred *anti* conformation for 5'-nucleotides in solution and also indicate that the presence of a possible hydrogen bond between the 2'—OH group

and the N₃ position in purine nucleotides has little effect upon the ring-chain interaction.

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II. SEQUENCE EFFECTS IN THE HIGH-RESOLUTION NMR SPECTRA OF OLIGONUCLEOTIDES.

A. OLIGOADENYLATE SPECTRA

Frank E. Hruska, Kimiko Umemoto, and Steven S. Danyluk

PURPOSE AND METHODS

In a previous report⁽¹⁾ the initial results of an NMR study of intramolecular interactions in several diribonucleoside diphosphates were reported. It was shown that the signals for base-ring protons in ApA-cyclic-p* were shifted significantly upfield relative to the corresponding mononucleotide signals. This upfield shift can be attributed to the shielding influence of ring currents on adjacent adenine rings in the base-stacked conformation, i.e., the dimer form in which the bases are stacked with their planes nearly parallel. Similar characteristic trends in signal patterns and chemical shifts have been reported recently for other diribonucleoside monophosphates.^(2, 3) These results raise the possibility of using high-resolution NMR spectroscopy for the determination of structure and sequences in short-chain nucleic acid segments. In order to test this possibility further, we have studied the sequence dependence of the proton spectra for the series of oligoadenylates (Ap)_nA-cyclic-p from *n* = 2 to *n* = 8. A summary of the pertinent results is given in this report.

PROGRESS REPORT

The high-resolution resonance spectra of the oligoadenylates (obtained from Miles Laboratories) were measured in D₂O (pD = 7.5) at 32°C with a Varian DA-60 spectrometer operating in the internal-lock mode. Because of the low concentrations of the oligoadenylate solutions (~0.02 M in monomer concentra-

tion), it was necessary to use a signal-averaging computer to improve the signal-to-noise ratio.

An illustration of the type of spectra obtained for several of the oligoadenylates is given in Figure 26, while the chemical shifts for the most prominent base-ring proton signals in the complete series of adenylates are shown in Figure 27. For ApA-cyclic-p, the presence of four signals confirms the nonequivalence of the two adenine rings. These signals can be assigned to H₂ and H₈ protons on the basis of deuterium exchange measurements. It has been shown by other workers⁽⁴⁾ that the H₈ protons in purine nucleotides exchange with deuterium much more rapidly at elevated temperatures (80°C) than H₂ protons. A similar behavior is observed for the oligoadenylates. Thus, heating of the dimer in D₂O at 85°C for 20 minutes results in a marked decrease in intensity of the two signals at *lowest* field. Hence, these signals are due to the two H₈ protons. The remainder of the assignment can be made from a comparison of ring-proton shifts in a variety of purine dinucleoside mono- and diphosphates.⁽⁵⁾ On this basis the H₂ signal at highest field can be assigned to the Ap base, while the H₈ signal at lowest field is attributed to the pA-cyclic-p base.

A similar detailed assignment of the signals for higher oligoadenylates is not feasible at present because of the increasing number of overlapping bands. However, it is still possible to show by deuterium exchange measurements that the cluster of signals at low field is due to the H₈ protons in all cases.

From the present results it is apparent that the spectra for the oligoadenylates are highly individual

* Abbreviation used: ApA-cyclic-p = 5'-OH-adenylyl-(3' → 5')-adenosine-2',3'-cyclic phosphate.

FIG. 26.—Several representative oligoadenyate spectra in D_2O .

(particularly for the lower oligomers) with distinctive signal patterns and chemical shifts observed for dif-

ferent oligomers. These patterns can be analyzed for the smaller chain length oligomers to yield definitive information about the structure in solution. Such analyses are not feasible using other spectroscopic techniques.

Of additional interest is the observation that the signals for both sets of protons are shifted upfield with increasing chain length. A trend of this type would be expected if an intramolecular stacking of the base rings is operative in all of the oligomers. As the chain length increases, the probability of a base being in the stacked conformation also increases, and hence the observed signal, which is actually an averaged signal for bases rapidly interchanging between stacked and unstacked forms, shifts upfield. The presence of base stacking is further confirmed by preliminary temperature studies which show that the H_2 and H_8 proton signals shift *downfield* with increasing temperature for all of the oligoadenyates (see following report for a more detailed description of the results for ApA-cyclic-p).

CONCLUSIONS

From the foregoing results it is clear that high-resolution NMR spectroscopy is potentially a very powerful nondestructive tool for the analysis of structure and sequence of nucleic acids. This is particularly true in the case of short sequences ($n = 2$ to 5), where a complete and unequivocal analysis of the structure

FIG. 27.—Chemical shifts of oligoadenyates in D_2O

is possible. The chief drawbacks of this technique at present are the relatively large quantity of sample required (typically 0.05 mg) and the extensive overlapping of signals for higher oligomers ($n > 5$). Both of these drawbacks can, it is hoped, be overcome by working at much higher field strengths (e.g., 220 Mcps).

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III. THE EFFECTS OF TEMPERATURE ON THE NMR SPECTRA OF OLIGONUCLEOTIDES.

A. (Ap)₂-CYCLIC

Frank E. Hruska and Steven S. Danyluk

PURPOSE AND METHODS

A problem of considerable current interest in molecular biology is the nature of the forces which stabilize nucleic acids, particularly S-RNA, in a given conformation in solution. It has been suggested,⁽¹⁾ from theoretical considerations, that the conformational stability is derived primarily from a free energy decrease associated with the stacking of adjacent bases in an aqueous environment. This hypothesis is supported by recent optical (UV,^(2,3) ORD,⁽³⁾ CD⁽⁴⁾) and NMR⁽⁵⁻⁷⁾ studies (cf. preceding report) on a variety of model oligonucleotides. A number of attempts^(2,8,9) have also been made to derive thermodynamic data for the stacking process from optical measurements. Although such measurements have yielded self-consistent sets of ΔH , ΔF and ΔS values, the assumptions involved in interpreting the optical data are open to considerable question. It would, therefore, be desirable to test the validity of these parameters by a different procedure. Accordingly, we have investigated the stacking equilibrium for the dinucleotide, ApA-cyclic-p over a range of temperature by NMR. It has been shown in the preceding report that the shifts for the base-ring proton of this compound are located significantly upfield from the corresponding monomer shifts. If this upfield shift is the direct consequence of an intramolecular stacking of the adenine rings, then an increase in temperature should lead to a destacking and a *downfield* shift of the adenine signals.

PROGRESS REPORT

Solutions of ApA-cyclic-p (Miles Laboratories, Lot #2A14) were made up in D₂O at pD = 7.5 (phosphate buffer, ionic strength 0.1 M). The proton spectra of the solutions were measured with a Varian DA-60 spectrometer operating in the internal-lock frequency sweep mode and equipped with a Varian variable temperature probe and accessories. A Varian C-1024 time-averaging computer was used to enhance the signal-to-noise ratio of the spectra.

Figure 28 shows the proton spectrum for the base-ring protons at several temperatures, while Figure 29 illustrates the chemical shift-temperature curves over the entire temperature range studied. The assignment of the signals to individual bases has been discussed elsewhere.⁽⁷⁾ The results summarized in Figures 28 and 29 demonstrate clearly that the signals shift *downfield* with increasing temperature. Such a temperature dependence can only be interpreted on the basis of an intramolecular destacking of the dimer with increasing temperature. Other explanations, such as dissociation of intermolecular aggregates, have been ruled out by appropriate experiments.⁽⁷⁾ Although the exact conformation of the stacked dimer cannot be established from the chemical shift data alone, a consideration of the observed chemical shift-temperature curves (in particular the appreciably larger shift changes of the H₂ protons) favors a conformation similar to that proposed for ApA,⁽⁹⁾ i.e., nucleotides in the *anti* form with adjacent bases oriented at $\sim 30-45^\circ$ with respect to each other.

FIG. 29.—Plot of the chemical shift in cps of H_2 and H_8 of ApA-cyclic-p, relative to tetramethylammonium bromide, versus temperature.

An analysis of the chemical shift-temperature curves to yield thermodynamic parameters for the stacking process can be carried out by a procedure similar to that reported recently by Jardetzky.⁽¹⁰⁾ If it is assumed that ApA-cyclic-p exists as a simple equilibrium mixture of stacked and unstacked forms [i.e., ApA-cyclic-p (U) $\rightleftharpoons$ ApA-cyclic-p (S)] and that the enthalpy change for the stacking process, ΔH_s , is independent of temperature (in the range studied), then ΔH_s can be evaluated from the slope of the plot of

$$\left[\frac{1}{(\delta_2 - \delta_U)} - \frac{1}{(\delta_1 - \delta_U)} \right] \left[\frac{1}{T_2} - \frac{1}{T_1} \right]^{-1}$$

versus $(\delta_m - \delta_U)^{-1}$, where δ_2 and δ_1 are the observed shifts at T_2 and T_1 , δ_U is the shift in the unstacked form (i.e., at the leveling-off point) and $\delta_m = \frac{1}{2} (\delta_1 + \delta_2)$.

A satisfactory straight-line plot was obtained using the shift change for the H_2 proton of pA-cyclic-p. The ΔH_s value of -9.9 ± 2 kcal mole⁻¹ (298°K), although somewhat higher than values reported for ApA^(2,9) and pApA,⁽⁸⁾ is within the experimental errors of the different methods. The NMR results, accordingly, provide an independent test of thermodynamic results obtained from optical measurements.

CONCLUSIONS

An NMR study has been made of the effect of

FIG. 28.—Proton resonance spectra for the base-ring protons of ApA-cyclic-p at several temperatures; pH = 7.5; concentration = 0.01 M (monomer).

temperature upon the spectrum of the base-ring protons of ApA-cyclic-p. It is concluded from the observed shift changes that an appreciable fraction of the bases exist in a stacked conformation below $\sim 60^{\circ}\text{C}$. The fraction of stacked bases increases progressively with decreasing temperature, and a thermodynamic analysis of the data yields an enthalpy change of -9.9 kcal mole $^{-1}$ for the stacking process. This study shows that NMR is a useful adjunct to other techniques for studying conformational changes in oligonucleotides.

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CONFORMATIONAL CHANGES OF BIOPOLYMERS. PART III. PULSED NMR STUDIES OF CONFORMATIONAL CHANGES IN RIBONUCLEASE

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PURPOSE AND METHODS

In the past, conformational changes induced in proteins by heat, pH changes, and salt effects have been followed by optical and/or hydrodynamic techniques. Such measurements have yielded valuable thermodynamic data relating to the conformational properties of proteins in different equilibrium states. However, the techniques used do not give any information about the dynamics of these conformational changes. The objective of this study is to obtain data pertaining to the dynamics of structural changes for proteins and nucleic acids.

The technique adopted for this study, pulsed NMR spectroscopy, involves the measurement of the nuclear spin-lattice relaxation time, T_1 , of protons on the biopolymer. Such measurement provides a more direct measure of the inter- and intramolecular mobility of the polymer than other NMR (CW)^(1,2) or optical techniques.⁽³⁾ Accordingly, as part of a continuing NMR study of conformational properties of biopolymers, we have investigated the effect of thermal change upon the proton relaxation time of ribonuclease (RNase).

PROGRESS REPORT

In any study of relaxation times of protein solutions, a major complication is presented by the presence of

large numbers of solvent protons. This problem can be circumvented, however, by exhaustive dialysis of the biopolymer solution in D_2O to remove both the solvent protons and exchangeable protons on the biopolymer. For such solutions, the measured relaxation time will then be due to residual protons of the biopolymer. Any changes in inter- and intramolecular mobility of the biopolymer, induced by pH or temperature changes, will be reflected by alterations in the T_1 value. In the present case RNase (bovine pancreatic $5 \times$ recrystallized, Nutritional Biochemicals, lot #3183) was dissolved in D_2O (USAEC, 99.8% isotopic purity in deuterium), and the solution was then dialyzed against D_2O at 4°C until the signal area for the high-resolution HDO was the same as for 99.8% D_2O . As a result of this procedure, a 10% (by weight) protein solution has a ratio of protein proton: solvent proton of greater than 20:1. On this basis, the residual solvent protons will not contribute significantly to the measured T_1 value. The T_1 values were measured by conventional pulsed NMR techniques.⁽⁴⁾ A summary of the spin-lattice relaxation times for ribonuclease solutions at two different concentrations and over the temperature range 24° to 95° is given in Figure 30. Also shown in Figure 30, for comparative purposes, is the temperature dependence of the relaxation time for a D_2O solution

FIG. 30.—Variation of spin-lattice relaxation time, T_1 , with temperature; O, 50 mg/ml ribonuclease in D_2O ; □, 100 mg/ml ribonuclease in D_2O ; △, 100 mg/ml amino acid mixture in D_2O .

containing a mixture of the constituent amino acids of ribonuclease made up to a concentration of 100 mg/ml; the proportion of each amino acid corresponds approximately to that in ribonuclease. The results in Figure 30 indicate that the T_1 values for protein protons are much shorter than those for protons of amino acids over the entire temperature range. Similar results were obtained previously for synthetic organic polymers.⁽⁶⁾ The shorter relaxation times in this protein are consistent with a much slower mobility of its amino acid residues.

The T_1 values for both ribonuclease solutions are constant with an increase in temperature up to approximately 60°C, where a sharp increase is noted in a relatively narrow temperature range (60°–70°C). Such abrupt transitions in relaxation time are not generally observed for polymers at their melting or glass transition temperatures.^(6, 7) Note, however, that the T_1 transition, centered at 66°C, occurs in the same temperature range as transitions observed in the UV (62°C),⁽³⁾ and high-resolution NMR spectra (60°–70°C)⁽⁸⁾ for aqueous solutions under similar conditions. The latter spectra have been attributed to a thermally

induced unfolding or loosening of the compact native structure of the protein. The present T_1 measurements agree with this interpretation and, in addition, provide a rough estimate of the relative increase in local segmental and side-chain mobilities which accompanies the unfolding process. An approximate time-scale for these motions can be obtained from the magnitude of correlation times, τ_c , derived for the case of nearest-neighbor pair interactions.⁽⁹⁾ For a 100-mg/ml solution of ribonuclease, τ_c is calculated to be 3.14×10^{-10} sec at 40°C and 1.21×10^{-10} sec at 90°C (τ_c for the amino acid solution at 40°C is 0.17×10^{-10} sec). The correlation times for the protein solutions are considerably greater than correlation times for solutions of small molecules, where τ_c values are typically $\sim 10^{-12}$ sec⁽⁵⁾ but are similar to those for other polymers.^(6, 7) Since τ_c is inversely related to the rate of the motional process(es), the mobilities of local regions of the protein molecule accordingly increase by almost threefold in the transition region. The activation energy associated with the change is calculated from τ_c values to be 12 ± 1.5 kcal mole⁻¹.

CONCLUSIONS

These results demonstrate that pulsed NMR measurements can yield significant data relating to the conformational properties of proteins in solution. In particular, it has been possible to obtain a relative time scale for the motional changes which occur in the denaturation process. Application of this technique to other biopolymer systems will test this relationship further.

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CORRELATION OF THREE-DIMENSIONAL CONFORMATIONS WITH AMINO ACID SEQUENCES OF PROTEINS

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PURPOSE AND METHODS

In the 1966 Annual Report⁽¹⁾ we described two-dimensional projections, called "helical wheels," to divide the amino acid sequences of proteins into categories with helical and nonhelical *potential*. This structural division substantially reduces the number of conformations a protein can assume. The number can be decreased still further by imposition of chemical and X-ray diffraction information. The quantity of ancillary information required to deduce an accurate and unique conformation, however, is not clear. This problem, examined in this report, is of special importance in those cases where the proteins either cannot be crystallized or are otherwise not suitable for X-ray analysis.

Because our knowledge is limited to results from only a few proteins, we chose to investigate tobacco mosaic virus (TMV), for which both chemical and X-ray diffraction data are available. The complete sequences of the 158 amino acid residues in two strains (*vulgare* and *dahlemense*) are known, and the amino acid replacements have been identified in many mutants.⁽²⁻⁴⁾ With X-ray diffraction techniques, oriented gels have been used to obtain fiber diagrams from which the symmetry of the virus and the size and shape of its individual subunits have been deduced.⁽⁵⁻⁸⁾ Unfortunately, the lack of either single crystals or suitable isomorphous derivatives has, thus far, made it impossible to determine the conformation of either the protein or the RNA in each subunit.

Caspar⁽⁹⁾ and Franklin^(5, 10) calculated the radial distribution of electron density. They related this distribution to the contributions of both the protein and the RNA. In 1963, Holmes and Klug⁽⁸⁾ provided a more detailed projection of electron density at a resolution of 10 Å. In the areas of disagreement, we tentatively assumed that the more recent projection obtained by Holmes and Klug superseded the earlier calculations by Caspar⁽⁹⁾ and Franklin.^(5, 10) To construct our model of TMV protein, we combined the X-ray diffraction results with the use of helical wheels⁽¹¹⁾ and auxiliary chemical data. The estimated helical percentage of about 40%⁽¹¹⁾ suggests that the application of methods designed to distinguish helical from nonhelical segments should be effective.

The TMV protein was first represented by a simple and inexpensive flexible model, consisting of electrical tubing (spaghetti-type) and rubber tubing. Both the

length of the electrical tubing and the diameter of the rubber tubing were selected to fit a scale of $\frac{1}{8}'' = 1 \text{ Å}$. It was assumed that the polypeptide chain in nonhelical segments was in an extended form corresponding to approximately 3.5 Å of length per amino acid residue. Each peptide unit in these segments was represented by black rubber tubing equivalent to 4 Å in diameter. Side-chains of polar residues were generally omitted, but those of hydrophobic residues were constructed of plastic beads and tied to the rubber tubing as needed. Cylinders of white rubber tubing, equivalent to 10 Å in diameter to include side-chains, were used to represent the six segments previously predicted to be helical. Each was assumed to be an α -helix, with 3.6 residues per turn and a pitch of 1.5 Å per residue. The approximate alignment of the helices was predicted from the electron density maps.^(5, 8)

Spatial relations among the various segments were determined with the aid of ancillary information, such as the molecular dimensions.⁽⁵⁾ The known shape of the subunit was outlined on cardboard to the same scale as that used for the flexible model. The radial distances

FIG. 31.—Model of TMV protein, top and side views. (Drawings by Mrs. Kathryn R. Ely.)

were marked off at intervals of 10 Å, and the flexible model was fitted to the shape. Wherever possible the hydrophobic side-chains were turned away from the surfaces exposed to solvent and directed toward the inside of the molecule.^(12, 13)

PROGRESS REPORT

Drawings of two views of the completed model of the polypeptide chain in one subunit are presented in Figure 31. The proposed helical segments, numbered 1 to 6 in order of alignment from amino to carboxyl end of the protein, are represented by cylinders. Individual side-chains are not shown in either the helical or non-helical segments. The radial distances from the axis of the virus particle are listed below each drawing. The outlines indicate the maximum amount of space that can be filled by each subunit. In the vicinity of 40 Å the protein segments are in contact with the RNA of the virus, but the RNA is not shown in the drawings.

The single cysteine residue in the protein is located in helix 2 at a radius of 56 Å. This residue lies in a hydrophobic region and is inaccessible to bulky sulfhydryl reagents. In an attempt to identify the two pairs of carboxyl groups believed to be involved in the binding of lead, the model was scanned in the vicinity of the known binding sites, at radii of 25 and 84 Å. If the model is correct in these regions, the residues in question are probably Asp-64 and Asp-66 in the first site and Glu-131 and Glu-145 in the second. A further consequence of the model is the identification of the protein segments bordering on the sites occupied by the RNA. Unequivocal assignments of the positions of the N- and C-terminal residues are still not possible, and they have arbitrarily been placed at radii of about 42 Å (N-terminus) and 67 Å (C-terminus).

CONCLUSIONS

The type of flexible model devised to represent the structure of TMV has proved useful in studies of overall conformations of proteins in general. Since the lengths and diameters of the amino acid residues in the helical and nonhelical segments are reduced to average values, the models can be rapidly constructed and oriented in the desired conformations. It should be emphasized, however, that the flexible models are not designed for detailed investigation of specific regions of the protein molecules. For this purpose we employ the Corey-Pauling-Koltun (CPK) models, supported on a lattice-work of 1/2" rods.

Large quantities of auxiliary chemical and X-ray

information are required for the completion of accurate models of the three-dimensional structures of proteins. While this information is not sufficiently detailed in the case of TMV, the present model does represent a plausible and approximate fit to the experimental results. This model and its successors can also be used in the design of chemical experiments. Included in the latter category are attempts to test the tentative assignments of the carboxylate pairs in TMV and the binding sites for RNA in the tobacco mosaic virus.

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ISOLATION AND CHARACTERIZATION OF MITOCHONDRIAL DNA OF TETRAHYMENA

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PURPOSE AND METHODS

Several recent papers have reported the presence of DNA in mitochondria of the ciliated protozoan, *Tetrahymena*.⁽¹⁻³⁾ The isolation of mitochondrial DNA from *Tetrahymena* cells has also been reported,⁽³⁻⁵⁾ and its buoyant density has been shown to differ from whole-cell DNA. Suyama and Preer,⁽³⁾ however, found a value of 1.682 g cm^{-3} for the buoyant density of mitochondrial DNA, while Parsons⁽⁴⁾ found a value of 1.671 g cm^{-3} . Although a strain difference might account for the apparent discrepancy, the possibility of confusion from contaminating polysaccharides⁽⁶⁾ in the latter case was likely.

The development of the technique for visualizing DNA strands under the electron microscope^(7, 8) has made it possible to examine mitochondrial DNA from *Tetrahymena*. Mitochondrial DNA's from various

metazoans have been seen under the electron microscope. Although all types of mitochondrial DNA preparations are reported as small ($5\text{--}6 \mu$) and circular in conformation,⁽⁹⁻¹²⁾ the existence of circular mitochondrial DNA in the unicellular organism, yeast, recently has been challenged.⁽¹³⁾

We wished to determine whether a real difference exists in size and conformation between protistan and metazoan mitochondrial DNA's. With this question in mind, we decided to reinvestigate the nature of *Tetrahymena pyriformis* (W) mitochondrial DNA.

PROGRESS REPORT

Method of Isolation of Mitochondrial DNA

Mitochondria were isolated and purified from whole-cell homogenates of *Tetrahymena* by the method of Kobyashi.⁽¹⁴⁾ These mitochondrial preparations were

FIG. 32.—Electron micrograph of a typical preparation of purified mitochondria fixed in veronal acetate buffered 1% OsO_4 , at pH 7.7. $\times 20,000$.

ULTRAVIOLET ABSORBANCE

FIG. 33.—Densitometer tracings of ultraviolet photographs of *Tetrahymena pyriformis*(W). A, Whole-cell DNA; B, mitochondrial DNA; and C, mitochondrial DNA heated for 10 min at 100°C and rapidly cooled. The DNA samples were centrifuged for 20 hr at 25°C at 44,700 rpm. The starting density of the CsCl solution was 1.700 g cm⁻³. Salmon sperm DNA ($\rho = 1.705$ g cm⁻³) was used as a marker. Peaks of density 1.692 g cm⁻³ and 1.714 g cm⁻³ are from native and denatured whole-cell DNA, respectively. Peaks of density 1.683 g cm⁻³ and 1.706 g cm⁻³ are from native and denatured mitochondrial DNA, respectively.

FIG. 34.—Electron micrograph of mitochondrial DNA filaments spread by the loop method. Note the frequent breaks along the lengths of several filaments. $\times 12,000$.

examined in the electron microscope to identify them conclusively and to determine whether contaminating material was present. The electron micrograph shown in Figure 32 is typical of such mitochondrial preparations. In practice, 10 liters of *Tetrahymena* culture, at 10^5 cells/ml, were harvested, and the mitochondrial pellets were resuspended in 5.0 ml of *p*-aminosalicylic (6% w/v)–0.2 *M* sodium arsenate, at pH 8. Under these conditions, the mitochondria swell but do not lyse. To release the DNA, these mitochondria were lysed by the addition of sodium deoxycholate (0.5%), and the clear lysate was phenol-extracted to remove the protein. After exhaustive dialysis against $0.1 \times$ SSC ($0.15 \text{ M NaCl} + 0.015 \text{ M sodium citrate} = 1 \times$ SSC), the RNA was removed by ribonuclease treatment, further phenol extraction, and dialysis. The yield of DNA from this extraction ranged between 0.5 and 1.0 mg per 10^9 cells.

Buoyant Density of Mitochondrial DNA

The mitochondrial DNA preparations were examined in the analytical ultracentrifuge (Model E), and their buoyant density was determined by isopycnic

FIG. 35.—The contour length distribution plot of a mitochondrial DNA preparation showing bimodal distribution of filament lengths.

FIG. 36.—The contour length distribution plot of an aged mitochondrial preparation with the majority of filaments being less than 2 μ in length.

centrifugation in a CsCl density gradient. Figure 33A presents densitometer tracings of UV-absorption profiles of whole-cell and mitochondrial DNA. The buoy-

ant density of mitochondrial DNA is 1.683 g cm^{-3} , which is lower than whole-cell *Tetrahymena* DNA ($\rho = 1.692 \text{ g cm}^{-3}$), see Figure 33B. Also, there are no other UV-absorbing bands. Figure 33C shows that heat denaturation of the mitochondrial DNA results in a shift in the buoyant density to a denser species ($\rho = 1.706$), suggesting the double-stranded nature of this DNA.

Electron Microscopy

Purified *Tetrahymena* mitochondrial DNA was prepared for electron microscopy by the technique of Freifelder and Kleinschmidt.⁽¹⁵⁾ For our study, DNA (5–10 $\mu\text{g/ml}$) in 1 M ammonium acetate, plus 0.5% neutralized formaldehyde containing 0.01% cytochrome C, was either directly looped onto gold parlodion-coated grids, or was floated onto a solution of 0.3 M ammonium acetate plus 0.5% formaldehyde, and then was picked up on grids. The DNA was stained according to the uranyl acetate method of Wetmur, Davidson, and Scaletti.⁽⁸⁾

Electron micrographs showed a predominance of rods (Figure 34), when the DNA solutions were looped directly onto grids. The contour length distribution in

FIG. 37.—Electron micrograph of degraded mitochondrial DNA filaments. Length measurements from this preparation showed the filaments to be shorter than 1 μ . $\times 24,000$.

FIG. 38.—Electron micrograph of a mitochondrial DNA preparation spread by the flotation method. Note the presence of open circular filaments and highly collapsed or twisted filaments. $\times 24,000$.

Figure 35 appears to be bimodal, with one peak at 6μ and one at 12μ .

Figure 36 presents a size distribution plot of rods of an older mitochondrial preparation, with the majority of filaments being less than 2μ in length. In the same preparation, approximately 4% (4/100) of the sample was in the form of circles, ranging in size from 0.3 to 2μ .

The preparation shown in Figure 37 was treated with RNase twice and was stored for a longer length of time than any of the other preparations. In this sample no circles were found, and all lengths were shorter than 1μ .

The flotation method produced a different proportion of rods to circles. Here, the number of circles increased (Figure 38) with many of them being either in a highly collapsed or twisted form that was too difficult to measure. The contour lengths of measurable circles ranged from 2 to 12μ .

The presence of circles and the length distribution appear to depend upon how the preparation is applied to the grid (by loop or flotation), the extent of chemical treatment, and the age of the DNA stock solutions. Nass⁽¹⁰⁾ pointed out the possibility of fragmentation

of originally circular molecules, since increasing proportions of linear fragments were found in her DNA samples after prolonged storage.

CONCLUSIONS

This report confirms the growing list of cases that demonstrate the occurrence of a unique DNA in mitochondria from *Tetrahymena*.^(3, 4) The buoyant density of our mitochondrial DNA ($\rho = 1.683 \text{ g cm}^{-3}$) is in good agreement with the value reported by Suyama and Preer.⁽³⁾ The density shift of 0.033 g cm^{-3} on heat denaturation of mitochondrial DNA is consistent with its being a double-stranded molecule. The absence of contaminating polysaccharides and other ultraviolet-absorbing density species in the CsCl density gradient profiles, as well as the electron microscopic examination of mitochondrial preparations, attest to the purity of our mitochondrial DNA preparations.

Mitochondrial DNA from *Tetrahymena*, examined by electron microscopy, consists of both rods and circles. Other investigators,^(9, 10, 13) also have found both kinds of DNA filaments in their preparations.

Questions about the shearing of DNA and the dif-

ferential susceptibility to fragmentation of circles, versus rods, are of paramount importance.^(9, 10) The bimodal distribution of contour lengths in relatively unsheared DNA preparations applied to the grid by the loop method suggests an upper limit of 12 μ for the length of rods, which have a tendency to break into half-molecules.⁽¹⁶⁾ Circles seen by flotation also have an upper limit of contour length of about 12 μ . A number of possible alternative explanations suggest themselves: 1) open-ended rods and circles naturally coexist, and 2) rods and circles can be derived from each other. The presence of breaks in extended DNA filaments, the exclusive presence of small circles in certain preparations applied to the grids by the loop method, and the progressive shortening of filament lengths with storage we take as an indication of the extreme fragility of both circles and rods as filament length increases.

The estimated size (12 μ) of the mitochondrial DNA filaments, rods and circles, in *Tetrahymena* is larger than any DNA reported so far. Suyama and Preer⁽³⁾ have estimated that the DNA content of a *Tetrahymena* mitochondrion is equivalent to a molecular weight of 240×10^6 , if it were all in one molecule of DNA. It was proposed recently that a mitochondrion contains several DNA molecules.⁽¹⁰⁾ If the molecular weight estimate of Suyama and Preer⁽³⁾ is correct, the data presented here would suggest that each mitochondrion may contain up to 10 molecules of DNA with a molecular weight of 2.4×10^7 .

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DESIGN OF A MULTIUNIT AUTOMATIC SAMPLING SYSTEM

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PURPOSE AND METHODS

In an investigation of rhythms in the growth cycle of *Tetrahymena pyriformis*, a system was devised to insure controlled temperature, illumination, and automatic collection of samples of from one to four independently grown cultures, at given time intervals during the growth cycle. This system provides reliable data on population growth kinetics of batch cultures under a wide range of temperature and illumination

conditions. A single-unit, automatic sampling device was described previously.⁽¹⁾

PROGRESS REPORT

Design of the Sampling System

The growth flask designed for this purpose is a modified 2.5-liter Pyrex low-form culture flask. (See Figure 39 for design of system.) Modifications of the flask, seen in Figure 40, include (1) an elongated neck,

allowing the flask to extend out of a water bath, (2) a central mixing chamber, which accommodates a Teflon-coated magnet, (3) a narrow glass finger, protruding into the flask, which holds a thermocouple wired to a Honeywell potentiometer that monitors the temperature of the medium, and (4) an inlet hose connector for air, and an outlet hose connector, for taking samples, located around the largest perimeter of the flask. The outlet connector terminates in a luer tip to accommodate a No. 15 hypodermic needle. The inlet connector includes a short capillary tube projecting into the flask. Air is bubbled through sterile distilled water and is filtered through cotton before it passes into the flask. A series of valves, the last of which is a Whitey micrometer control valve, controls the air flow, which is monitored by a spherical float flowmeter (flow rates $\sim 25\text{--}85$ cc/min).

The water bath is a 4.5-gallon cylindrical Pyrex jar supported by a large tripod (Canalco). The flask is stabilized in the bath by a collar of lead rings. A Bronwill heater-circulator maintains the water at a constant temperature. To achieve temperatures below room temperature, a Schuco cooling probe is also immersed in the water bath. Directly below the water bath is a magnetic stirrer with its mechanical parts inverted to enhance heat loss.

Bright illumination is provided by 15-watt cool, white, fluorescent Circline lamps (Sylvania) located either directly below the flask or both above and below the flask. These lamps are connected through a ballast box to a Sangamo timer, which permits a wide choice of light-dark regimes. Fluorescent lights, mounted on the ceiling, provide constant dim illumination ("moonlight"). The flask, the water bath, and the lamps are enclosed in a compartment which minimizes extraneous light. Four growth compartments allow a maximum of four flasks to be run simultaneously.

The outlet hose connector of each flask is connected, by way of a No. 15 needle through 16-gauge polyvinyl chloride tubing, to a Filamatic automatic pipetting machine. The polyvinyl tubing is adapted to include a short length of rubber tubing, which passes through a normally-closed solenoid-operated clamp. Just before entering the intake valve of the Filamatic, the polyvinyl tubing passes through a minimal-volume four-way glass connector, which joins the tubing from all four flasks. Figure 41 shows this connector, the tubing, and the luer-tip adaptors used in the system.

The master control of the automatic sampling system is a Zenith timer, which has adjustable time tabs

FIG. 39.—One of four growth compartments and the other components of a multiunit automatic sampling system. GC, growth compartment; FM, flowmeter; P, potentiometer; ZT, Zenith timer; SC, solenoid control box; TI, interval timer; FR, Fractomat; FI, Filamatic; C, minimal-volume four-way connector; SW1, SW2, microswitches; S1, solenoid operated clamp; ST, Sangamo timer.

FIG. 40.—Modified low-form culture flask and Mariotte bottle. In practice, the Mariotte bottle is positioned higher and a solenoid-operated clamp, similar to S1 in Figure 39, is located in the S curve of the rubber tubing.

that trigger events at intervals of 15 min. In the experiments described below samples are taken from each flask every 90 min. The Zenith timer, through relay RY1 (Figure 42), operates an ATC interval timer T1, which determines the length of the sampling time. The Zenith timer also actuates a solenoid and the Filamatic, through relays RY2 and RY4. When the interval timer completes its operation, microswitch SW1, mounted on the case of the Filamatic, is opened by

contact with the eccentric arm of the Filamatic as it completes its last stroke. This closes the solenoid-operated clamp and stops the Filamatic. The stepping-relay, RY2, sequentially actuates solenoids S1, S2, S3, and S4, so that the next event of the Zenith timer results in a sample being collected from the next flask. Relay RY3 resets relay RY2 to the No. 1 position when selector switch SW3 is operated.

The aliquots delivered by the Filamatic are collected by a Fractomat automatic fraction collector into test tubes that contain a Coulter Counter electrolyte medium (2% formalin in 0.6% NaCl). The Fractomat is operated in the drop collection mode, with the input provided by microswitch SW2, which is mounted on the case of the Filamatic. The microswitch is activated upon contact with the eccentric arm of the Filamatic at each cycle of the syringe. An event marker records each advance of the Fractomat, by a side-arm pen mounted on the Honeywell potentiometer. Five aliquots are withdrawn from each flask during each sampling period. The contents of the first three tubes are discarded, to allow for clearance of the fluid from the previous sample. The contents of the last two tubes are counted on a Coulter Counter (Model B).

As a modification of the automatic sampling system, an automatic, programmed-feeding device has been developed. This device provides turbidostat-like conditions for batch cultures. Dual-sequence program timers control a solenoid-operated clamp, which allows the periodic flow of medium from the Mariotte bottle to the growth flask (Figure 40), following the program prescribed by demand feedings in a turbidostat.⁽²⁾

Operation of the Sampling System

Figure 43 shows data from a set of experiments that illustrate the capacity of the sampling system to produce reliable data on the population growth kinetics

FIG. 41.—Connectors, adaptors, and tubing used in the system. A, rubber tubing; B, luer-tip adaptor; C, No. 15 needle; D, polyvinyl chloride tubing; E, minimal-volume four-way connector.

FIG. 42.—Circuit diagram of the multiunit automatic sampling system.

FIG. 43.—Growth curves of batch cultures of *Tetrahymena pyriformis* at three temperatures.

f three batch cultures of *Tetrahymena* at three different temperatures (15, 20.5, and 27°). The generation times (GT) during the exponential growth phase at 27, 20.5, and 15° are strongly and inversely tempera-

ture-dependent, being 3, 4, and 6 hr, respectively (i.e., ultradian),⁽³⁾ and agree well with previously published values.⁽⁴⁾

At the end of the exponential ultradian growth phase, there is a phase of slow growth; this is clearly not a long plateau or stationary phase. The generation times in this growth phase are characteristically infradian (i.e., GT greater than 24 hr) and are without dramatic temperature dependence. These curves are smooth, indicating a lack of synchrony of cell division and a random mitotic index.

Manipulation of the lighting conditions during the infradian growth phase sometimes, but not always, results in the synchronization of *Tetrahymena* cell division.⁽²⁾ A switch-down in light intensity (LL → DD), at the times indicated in Figure 43, had no *lasting* effect on the rate of cell division in these particular experiments, although a correlated transient change (arrows) in the growth rate occurred.

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Cellular Biochemistry

FURTHER OBSERVATIONS ON THE BIOCHEMISTRY OF BROWN ADIPOSE TISSUE OF THE GROUND SQUIRREL

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PURPOSE AND METHODS

In the previous annual report,⁽¹⁾ data were presented that showed that mitochondria isolated from axillary brown adipose tissue of the euthermic ground squirrel (*Citellus tridecemlineatus*) differ from mitochondria of other tissues in several important respects. These include (a) decreased sedimentability, (b) abnormal swelling characteristics, and (c) impaired capacity for esterification of inorganic phosphate, despite a much higher concentration of respiratory enzymes than are found in liver mitochondria. Recent reports from other laboratories have shown that oxidative phosphorylation is markedly reduced in mitochondria isolated from brown fat of nonhibernating species (rats⁽²⁾ and rabbits⁽³⁾).

The present report is concerned with additional observations on the enzymatic properties of brown fat mitochondria, and with an extension of the previous experiments to include studies on hibernating and newly-aroused ground squirrels.

Most of the procedures employed were described in the previous report.⁽¹⁾ Many of the ground squirrels were used simultaneously in another experiment involving the injection of sheep red cells, in which only the spleen was of interest, so that it was advantageous to splenectomize some of the treated animals at the appropriate time during the immunologic study, then kill the animals at a later time for the experiments with brown fat or liver.* Newly-aroused animals were transferred from the cold room (5°C) to room temperature, and a thermistor was implanted subcutaneously in the region of the neck.† The ground squirrels were allowed to arouse spontaneously, and were killed when their body temperature had been stable for 15 min (34–38°C). The process required about 90 to 120 min.

* We gratefully acknowledge the cooperation of Dr. Bernard N. Jaroslow for placing these animals at our disposal.

† We are indebted to Mr. William J. Eisler for preparation of this apparatus.

PROGRESS REPORT

Fatty Acid Oxidation

It was previously reported that mitochondria from brown fat possessed only limited capacity (about $\frac{1}{10}$ that of liver mitochondria) to oxidize octanoate. We have subsequently found that replacement of octanoate by palmitate and fortification of the incubation medium with GTP, CoA, and carnitine permit vigorous oxidation (Table 2). There was no accumulation of acetoacetate in the medium.

There were no significant differences among mitochondria from euthermic, hibernating, and aroused animals with respect to rate of palmitate oxidation, although mitochondria from one newly-aroused animal showed over twice as much oxidative activity as the average.

Oxidative Phosphorylation

Table 3 presents a summary of work carried out on the phosphorylative capacity of mitochondria from brown fat of euthermic, hibernating, and aroused animals. Mitochondria from aroused animals displayed a somewhat higher rate of oxidation of all substrates. However, the phosphorylative efficiency was equally low in all cases; only the substrate-level phosphorylation associated with oxidation of α -ketoglutarate could be detected.

Various additions to the incubation medium were

TABLE 2. OXIDATION OF PALMITATE BY MITOCHONDRIA ISOLATED FROM GROUND SQUIRREL BROWN FAT

State of ground squirrel	No. of experiments	μ atoms O/hr/mg N	
		Average	S.E.
Euthermic	6	26.0	4.6
Hibernating	4	22.6	3.6
Aroused	5	27.9	2.6

TABLE 3. OXIDATIVE PHOSPHORYLATION BY MITOCHONDRIA ISOLATED FROM GROUND SQUIRREL BROWN FAT

Substrate	Euthermic			Hibernating			Aroused		
	No.	O/N ^(a)	P/O ^(b)	No.	O/N	P/O	No.	O/N	P/O
Succinate	6	22.5 ± 1.2	0.21 ± 0.13	3	14.9 ± 1.6	0.12 ± 0.10	4	37.1 ± 2.3	0.05 ± 0.01
α-ketoglutarate	7	15.7 ± 1.0	0.72 ± 0.07	7	15.2 ± 1.9	0.83 ± 0.05	5	25.6 ± 1.0	0.85 ± 0.03
α-ketoglutarate + NAD	1	20.4	0.70	1	11.6	0.84			
α-ketoglutarate + DNP	2	19.6	0.59	1	11.4	0.68			
β-Hydroxybutyrate	5	3.6 ± 0.6	0.07 ± 0.02	4	3.2 ± 0.3	0.22 ± 0.08	5	5.2 ± 0.1	0.35 ± 0.05
β-Hydroxybutyrate + NAD	1	11.0	0.25	1	4.1	0.28			
Fumarate	3	2.1 ± 0.6	0.05 ± 0.05						
Fumarate + NAD	1	6.3	0.0						
Glutamate				1	0.0	0.0			
NADH	1	16.2	0.03						
Ascorbate	1	19.0	0.07						

(a) O/N ratios: μ atoms O consumed/20 min/mg total N.

(b) P/O ratios: μ atoms P esterified/ μ atom O consumed.

FIG. 44.—Effect of Ca^{++} on accumulation of phosphate by liver and brown fat mitochondria of the ground squirrel. After 10-min incubation at 38 C, $8 \mu\text{M}$ of calcium chloride was added (0 time). LPr and LS, quantity of phosphate found in mitochondrial precipitate and supernatant, respectively, of ground squirrel liver; BFPr and BFS, similarly for ground squirrel brown fat. The initial quantities of orthophosphate (P_i) and of P_i plus ATP are shown for reference.

tried. These included insulin, bovine serum albumin, ultrasonically treated rat liver mitochondria, and various other fractions of rat liver mitochondria. None of these appreciably enhanced phosphorylative capacity.

Digitonin fragments prepared from brown fat mitochondria oxidized β -hydroxybutyrate at about the same rate as did fragments similarly prepared from liver mitochondria, but this oxidation was not accompanied by phosphorylation.

Effect of Massive Calcium Loading

In the presence of large quantities of Ca^{++} (0.004 M), mitochondria from liver, kidney, heart, or brain will accumulate calcium and phosphate in the same ratio (5:3) as hydroxyapatite when they are incubated in a medium similar to that employed for oxidative phosphorylation, and compounds that inhibit the latter also inhibit the former.

Figure 44 shows the results of an experiment in which mitochondria from brown fat and from liver were compared for their ability to accumulate inorganic phosphate (P_i) in the presence of Ca^{++} . In the case of liver mitochondria, upon the addition of Ca^{++} the amount of P_i in the supernatant decreased rapidly, whereas the amount recovered from the mitochondria rose rapidly. In the case of brown fat mitochondria, however, there was no accumulation of P_i in the mitochondria, but there was a steady increase of P_i in the supernatant resulting from the Ca^{++} -activated ATPase of the mitochondria.

The rate of oxidation of succinate by liver mitochondria was markedly increased upon the addition of Ca^{++} to about the same extent that it was increased by dinitrophenol. Brown fat mitochondria responded neither to Ca^{++} nor to dinitrophenol when respiring with succinate as the substrate.

TABLE 4. ENZYME DISTRIBUTIONS AMONG SUBCELLULAR FRACTIONS OF GROUND SQUIRREL BROWN FAT

State of ground squirrel	Fraction	Cytochrome oxidase		Succinate dehydrogenase		Malate dehydrogenase		Isocitrate dehydrogenase		Aconitase		Fumarase	
		% ^(a)	mM/mg N/min ^(b)	%	mM/mg N/min	%	mM/mg N/min	%	μM/mg N/min	%	μM/mg N/min	%	mM/mg N/min
Euthermic	Homogenate		5.15 ± 0.37		3.04 ± 0.55		9.18 ± 0.65		137 ± 15		527 ± 78		3.86 ± 0.40
	Mitochondria	72	9.14 ± 0.72	78	4.33 ± 1.35	22	6.50 ± 0.43	16	105 ± 2	84	1128 ± 101	80	6.38 ± 0.72
	Microsomes	23	9.19 ± 2.41	20	4.60 ± 1.49	18	25.3 ± 11.6	3	72 ± 5	6	251 ± 56	3	1.26 ± 0.84
	Supernatant	5	0.96 ± 0.26	2	0.07 ± 0.03	60	39.6 ± 18.5	82	1110 ± 440	10	200 ± 35	17	3.62 ± 0.55
Hibernating	Homogenate		4.85 ± 0.55		2.60 ± 0.41		8.32 ± 0.35		134 ± 10		447 ± 101		2.72 ± 0.11
	Mitochondria	77	7.59 ± 1.01	79	3.60 ± 0.58	18	6.12 ± 0.33	14	81 ± 14	79	656 ± 92	79	6.60 ± 0.33
	Microsomes	20	7.58 ± 1.80	20	4.39 ± 1.78	10	22.2 ± 10.8	6	107 ± 30	5	218 ± 103	3	0.86 ± 0.14
	Supernatant	3	0.45 ± 0.15	1	0.10 ± 0.03	72	33.3 ± 13.6	80	770 ± 205	16	325 ± 32	18	3.22 ± 0.15
Aroused	Homogenate		5.14 ± 0.48		5.66 ± 1.17						660 ± 72		
	Mitochondria	80	9.49 ± 0.46	78	5.95 ± 0.72					82	1322 ± 296		
	Microsomes	15	5.92 ± 0.84	17	4.72 ± 1.49					5	224 ± 89		
	Supernatant	5	0.79 ± 0.14	5	0.60 ± 0.19					13	294 ± 24		

^(a) Values are based on the activities recovered in the three fractions; in terms of the original homogenate, recoveries averaged about 95%.

^(b) Enzyme activities are expressed as amount of substrate consumed per minute per milligram total nitrogen, with standard errors.

TABLE 5. NITROGEN CONTENT AND SUBCELLULAR DISTRIBUTION IN GROUND SQUIRREL BROWN FAT

State of ground squirrel	mg N/g fresh weight of tissue	% N ^(a)			
		Nuclei (by difference)	Mitochondria	Microsomes	Supernatant
Euthermic	18.5 ± 0.79	26.5 ± 3.6	38.5 ± 4.7	12.0 ± 1.9	23.0 ± 1.8
Hibernating	24.3 ± 0.67	22.9 ± 4.9	43.5 ± 2.7	10.1 ± 1.9	23.5 ± 1.5
Aroused	19.9 ± 0.77	20.9 ± 1.2	40.0 ± 1.4	12.2 ± 0.9	26.9 ± 1.2

^(a) Values given are averages with standard errors.

Enzyme Distribution Studies

Differential centrifugation was used to study the distribution of a number of enzymes in brown fat. The results are shown in Table 4. It appears that malate dehydrogenase and isocitrate dehydrogenase are found in both the mitochondria and in the soluble phase of the brown fat cell. The remaining enzymes are probably localized exclusively in the mitochondria; however, some fragmentation of the mitochondrial membranes seems to have occurred in the course of preparation, so that soluble enzymes like fumarase and aconitase appear in the final supernatant, whereas cytochrome oxidase and succinate dehydrogenase, being insoluble, come down in the so-called microsomal fraction. This interpretation is consistent with the results of electromicroscopic examination of isolated brown fat mitochondria; an appreciable number of mitochondria showed loss of segments of both outer and inner membranes.*

*Electron microscopy was performed by Mr. Duane Haebeck.

In general, the specific activities of the enzymes tended to be highest in newly-aroused animals and lowest in hibernating animals, although few of the differences are statistically significant.

Table 5 shows the distribution of total nitrogen among the three fractions. The concentration of nitrogen per unit fresh weight of tissue is significantly higher in hibernating ground squirrels, although the distribution patterns are essentially the same, regardless of the state of the animals.

CONCLUSION

The evidence presented here, together with previous results from this laboratory,⁽¹⁾ show that the metabolism of mitochondria isolated from the brown fat of the ground squirrel is markedly different from that of tissues like the liver, but very similar to that of brown fat from nonhibernating species.^(2, 3) The concentration of respiratory enzymes is significantly higher, but the capacity to utilize the energy derived from the oxidation of various substrates is drastically

reduced. Attempts to supply factors isolated from other types of mitochondria that might couple phosphorylation to respiration have so far been unsuccessful; it is possible that the failure of brown fat mitochondria to synthesize ATP is the consequence of an atypical pattern of organization of the various components of the electron transport chain.

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BIOCHEMICAL CHANGES IN THE INTERSCAPULAR BROWN ADIPOSE TISSUE OF RATS DURING THE COLD ACCLIMATION PROCESS. EFFECT OF IONIZING RADIATION ON GROWTH OF BROWN FAT

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PURPOSE AND METHODS

The metabolic activity of brown adipose tissue in hibernating animals has long been of interest to physiologists and biochemists, but recently considerable attention has been paid to its role in nonhibernating

in the cold. The animals were killed by ether anesthesia, and the interscapular fat pad was carefully dissected away from white fat and muscle. The tissue was chilled, blotted, and weighed, then homogenized in 7 ml of a solution of 0.25 M sucrose and 0.0003 M

TABLE 6. CHANGES IN COMPOSITION OF RAT INTERSCAPULAR BROWN ADIPOSE TISSUE DURING EXPOSURE TO COLD ENVIRONMENT

Days at 5° C	Brown fat wt., mg			Total N, mg/g			PLP, μg/mg N			DNA, μg/mg N			RNA, μg/mg N			Cytochrome oxidase, mM/mg N/min			Succinate de- hydrogenase, μM/mg N/min			Malate de- hydrogenase, mM/mg N/min			Aconitase, μM/mg N/min		
	No.	Av.	S.E.	No.	Av.	S.E.	No.	Av.	S.E.	No.	Av.	S.E.	No.	Av.	S.E.	No.	Av.	S.E.	No.	Av.	S.E.	No.	Av.	S.E.	No.	Av.	S.E.
0	66	169	8	64	11.5	0.3	45	33.7	1.8	42	95	5	43	103	5	42	6.18	0.19	55	3.40	0.13	37	5.95	0.18	38	460	17
1	16	189	13	15	15.0	1.0	11	43.5	1.8	11	120	7	11	145	7	8	5.30	0.36	12	2.53	0.14	8	5.05	0.21	8	458	41
2	16	213	8	15	15.8	0.4	11	51.7	1.2	11	132	7	11	165	5	8	5.68	0.21	12	2.70	0.28	8	4.55	0.35	8	312	21
3	24	266	10	22	17.6	0.7	14	48.2	2.3	13	165	15	14	177	10	10	4.90	0.23	11	2.34	0.13	8	5.15	0.24	7	433	35
4	16	296	15	15	16.4	0.9	11	47.5	2.1	9	133	7	11	167	10	8	4.33	0.19	12	2.88	0.24	8	6.10	0.20	8	443	32
7	18	399	17	18	16.2	0.8	16	48.9	2.1	16	131	8	15	151	7	8	5.39	0.29	12	3.66	0.18	8	5.66	0.34	7	468	19
14	18	528	29	17	16.4	0.1	14	49.5	2.2	12	129	7	11	126	4	10	5.78	0.55	14	4.05	0.26	8	5.51	0.19	8	537	47
21	10	723	63	10	16.2	1.0	6	45.0	3.6	6	124	10	6	106	7	6	5.92	0.45	6	3.98	0.61	6	6.26	0.16	3	602	17
28	6	727	58	6	15.1	0.7	3	45.4	2.6	5	107	4	6	99	5	6	6.03	0.13	6	3.94	0.51	6	5.30	0.37	6	811	57
35	6	798	88	6	14.5	0.6	6	49.6	4.4	5	87	3	6	122	12	6	6.87	0.40	6	4.81	0.64	6	6.55	0.13	6	863	27
42	7	852	51	4	14.9	0.7	4	47.2	2.2	4	94	10	4	120	10	4	6.89	0.30	4	4.36	0.19	4	6.23	0.22	—	—	—

species, with particular reference to the growth of the tissue in response to exposure to low temperature.^(1, 2) In an attempt to define some of the changes occurring in brown adipose tissue during the process of cold acclimation, we have studied the interscapular brown fat of rats exposed for varying lengths of time to a 5° environment.

The rats used in these experiments were Sprague-Dawley males, usually 5 to 6 weeks old when placed

EDTA. Phospholipid and nucleic acids were measured in a portion of this homogenate according to the procedure of Munro and Fleck.⁽³⁾ Enzyme assay procedures were the same as those described in another report.⁽⁴⁾

Radiation effects on the growth of brown fat involved the exposure of a group of rats to 400 R 250-kVp X radiation, delivered at a dose rate of 200 R/minute. Most of the irradiated animals, along with the appropriate controls, were transferred immediately to the cold; some were kept at room temperature.

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FIG. 45.—Electron micrograph of interscapular brown fat of a control rat maintained at 24°C. (The magnification in Figures 45-49 is 20,000 $\times$.)

FIG. 46.—Electron micrograph of interscapular brown fat of a rat two days after transfer to 5°C

Fig. 47.—Electron micrograph of interscapular brown fat of a rat one week after transfer to 5°C

FIG. 48.—Electron micrograph of interscapular brown fat of a rat three weeks after transfer to 5°C

FIG. 49.—Same as Figure 48, different field. Note the unusual forms of mitochondria (arrows).

OBSERVATIONS

Growth and Composition

Table 6 shows the increase in fresh weight of brown fat and in total nitrogen per unit weight in the course of adaptation to 5° C. There was a steady increase in weight of the brown fat in the course of six weeks to a value almost 400% greater than the control weight; during this observation period the mass of brown fat of rats kept at room temperature increased in about the same ratio as the body weight. The concentration of total nitrogen reached a maximum after three days, remained constant with only minor fluctuations through three weeks, then declined slightly thereafter. The amount of total nitrogen in the entire fat pad increased progressively throughout the experiment, 2.0 mg at 0 time to 12.2 mg at six weeks.

The changes in concentrations of nucleic acids and of phospholipid phosphorus are also shown in Table 6. The maximum concentrations of both DNA and RNA were observed on the third day of exposure to cold. However, the total amounts of both continued to in-

crease beyond this time, even though the concentration decreased relative to total nitrogen; the total amount of brown fat DNA reached a maximum at about two weeks, then remained essentially constant, whereas the total RNA increased through the first five weeks.

The response of phospholipid phosphorus (PLP) was a 50% increase during the first two days, then virtually no change in concentration thereafter.

Enzymatic Activities

The changes in enzymatic activities are shown in Table 6. There was an immediate decrease in succinate dehydrogenase (SDH) activity, the values remaining below average for four days, then rising to a value significantly higher than the controls after two weeks. A slight increase was observed between two and six weeks, but none of the values during this interval differed significantly from one another.

In the case of cytochrome oxidase (CO), the initial decrease persisted considerably longer, and the concentration per unit nitrogen did not surpass the control levels until the fifth week of cold exposure. For both CO and SDH, the total amount of enzyme

FIG. 50.—Effect of 400 R total-body radiation on the weight of interscapular brown adipose tissue of rats placed at 5°C immediately after irradiation.

present in the tissue after six weeks of adaptation was more than seven times the initial amount.

The response of aconitase differed from that of the other two mitochondrial enzymes in that after the initial decrease in activity, there was a rapid return to normal values, with a progressive increase to a level at five weeks almost double that of the controls.

Malate dehydrogenase (MDH) activity also decreased during the first few days of cold exposure. However, although the activity returned to the control level within four days, there was no further increase such as was observed with the other enzymes. In the ground squirrel brown fat, only 20% of the MDH activity is associated with the mitochondria, the remainder occurring in the soluble fraction; hence, if one assumes that enzyme distribution patterns in the two species are similar, it is probable that changes in mitochondrial MDH could be masked by other changes in the soluble isozyme.

Electron Microscopy

Figures 45 to 48 are electron micrographs of representative fields of interscapular brown fat at different stages of cold-adaptation: control, two days, one week, and three weeks, respectively—chosen to emphasize the changes in mitochondria and lipid droplets. The mitochondria from the control sample resemble those observed in ground squirrel brown fat and show the characteristic tightly-packed cristae. Fat droplets are large and numerous, and many of them are in intimate contact with the mitochondria.

After two days in the cold, there is an increase in amorphous ground substance, a decrease in size of fat droplets, and a decrease in number of mitochondria per unit area. The mitochondria are more rounded and less distorted, possibly because they are not as tightly packed.

After one week, the mitochondria are relatively more numerous but appreciably smaller; the cristae seem better developed. The fat droplets are enlarged, relative to the two-day specimen.

There is not much difference between the three-week and the one-week animal, except for the presence in the former of numerous bizarre forms of mitochondria. Some of these are more apparent in Figure 49, which is from another field from the same animal from which Figure 48 was prepared. These unusual forms strongly suggest that the mitochondria are in the process of dividing.

Radiation Effects

Figure 50 shows that the increase in brown fat mass following cold exposure was appreciably depressed when rats were irradiated with 400 R immediately be-

FIG. 51.—Effect of 400 R on total DNA and RNA content of interscapular brown adipose tissue of rats placed at 5°C immediately after exposure.

fore transfer to the cold environment. There was no significant effect of radiation on the brown fat of animals kept at room temperature after exposure to 400 R.

It is difficult to assess the importance of anorexia and weight loss in the irradiated animals as far as hypertrophy of the brown fat is concerned. The irradiated animals lost about 8% of their initial body weight during the first two days, but thereafter gained weight at the same rate as the controls. However, during the period of two to seven days, when the increase in brown fat weight was roughly linear, the controls showed a rate of increase of 43 mg/day, whereas for the irradiated animals the rate was 33 mg/day.

Very few changes could be detected in nucleic acid concentration when the results were expressed in terms of total nitrogen. There was a consistent, although statistically insignificant, decrease in DNA/mg N between two and seven days. The total DNA and RNA contents of the tissue are shown in Figure 51.

Other measurements, including total nitrogen per unit weight of brown fat, PLP/mg N, and succinate dehydrogenase activity per mg N, indicated no essential differences in concentration.

Experiments are in progress to extend the time of observation beyond the two-week period used in these studies.

CONCLUSION

It is apparent that the increase in brown adipose tissue of rats exposed to a temperature of 5°C is the result of both hyperplasia and hypertrophy. The concentration of DNA rises rapidly to a maximum within three days, then decreases to control levels. However, the total amount of DNA in the tissue continues to rise for about two weeks; thus the early growth can be attributed primarily to an increase in cellularity, whereas subsequent increases in mass represent principally an increase in the size of the cells.

The protein content of brown fat increases sharply during the first two days, but this increase is not shared by the various enzymes for which we have assayed, all of which showed significant decrease during the first few days of cold exposure. The decrease in enzyme activities is reminiscent of the early changes

occurring in regenerating liver, when the rate of cell division exceeds the rate of mitochondrial duplication. The differences in the response patterns among cytochrome oxidase, succinate dehydrogenase, and aconitase probably reflect the different intramitochondrial distributions of these enzymes.

It is clear that after exposure to radiation the brown fat does not grow as rapidly as in unirradiated, cold-exposed rats, but more work needs to be done to establish the mechanism of this impairment. It seems likely, however, that inhibition of DNA synthesis plays a significant role.

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ELECTRONMICROSCOPIC EXAMINATION OF BROWN ADIPOSE TISSUE OF THE GROUND SQUIRREL

Douglas E. Smith,* Duane A. Habeck, Sharron L. Nance, and John F. Thomson

PURPOSE AND METHODS

A number of investigators have described the fine structure of brown adipose tissue, but for the most part they have studied tissues from nonhibernating animals such as the mouse, rat, or rabbit.⁽¹⁻⁴⁾ In hibernating species, such as the ground squirrel (*Citellus tridecemlineatus*), brown fat is of vital importance to the animal as the site of heat production during arousal from hibernation.⁽⁵⁾ The tissue possesses some unique metabolic characteristics, the most notable of which is the inability of the mitochondria to esterify inorganic phosphate (except at the substrate-level phosphorylation step of α -ketoglutarate oxidation).^(4, 6, 7) Since changes in the biochemical properties of brown fat take place during hibernation and arousal, an electronmicroscopic examination of brown fat from ground squirrels in different states of activity as undertaken to obtain information that might

be useful for correlation with enzymatic activity of the tissue.

Euthermic, hibernating, and newly aroused ground squirrels were examined. Small pieces of tissue from the axillary fat pads were placed in a 2.5% solution of glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) plus 0.06 M sucrose for 24 hr at 3°C, then in 1% osmium tetroxide for 8 hr. After dehydration with ethanol, the tissues were infiltrated with plastic (Epon 812, Araldite 6005, and dodecenyl succinic anhydride in a ratio of 5:4:12) in styrene according to the following schedule: 1:3 plastic:styrene for 1 hr, 1:1 for 3 hr, 3:1 for 10 hr, then plastic without solvent for 6 hr, followed by thermal polymerization.

Radioautography was undertaken on one newly-aroused animal. The ground squirrel was removed from the 5°C environment and allowed to awaken spontaneously. After 100 min, the animal was lightly anesthetized with ether, and 1 mCi of sodium palmitate-9,10-³H (500 Ci/mole) was injected intravenously. The ground squirrel was killed 30 min later

* This work was begun by Dr. Smith; preliminary observations were completed before his death on August 28, 1966.

FIG. 52.—Section of brown fat from a newly-aroused ground squirrel (15,000 $\times$)

FIG. 53.—Section of brown fat from a euthermic ground squirrel (20,000 $\times$)

FIG. 54.—Radioautograph of brown fat from a newly-aroused ground squirrel killed 30 min after injection of tritium-labeled palmitate (20,000 $\times$). Note the β -tracks within the lipid droplet. (The granular material in the upper and lower right-hand corners is an artifact.)

and the axillary brown fat removed and treated as described above.

PROGRESS REPORT

The first impression one gains from examining an electron micrograph of ground squirrel tissue is that the tissue seems to be composed principally of lipid droplets and mitochondria. Figure 52 is taken from an aroused animal, at a time when the lipid content of the tissue had been markedly depleted during the arousal process. Most of the mitochondria show very numerous cristae, many of which appear to traverse the entire mitochondrion. Very few other cytoplasmic components are visible; in other fields, particulates that resemble lysosomes and peroxisomes are occasionally seen, but very little endoplasmic reticulum is discernible.

The brown fat from the euthermic animal (Figure 53) shows much larger lipid droplets and consequently more compression and distortion of other cellular inclusions. The tissue from the hibernating animal (not shown) closely resembles that from the euthermic ground squirrel. Other differences between euthermic and aroused animals, not indicated in Figures 52 and 53, include much greater pinocytotic activity in the aroused animal, and more rounded and more centrally located nucleus.

Figure 54 is a micrograph of brown fat from an aroused animal given palmitate- ^3H . The temperature of the ground squirrel had reached normal before the palmitate was injected, and the depletion of the tissue lipid was thus maximal.⁽⁵⁾ Hence the 30-min period between injection of the tracer and the killing of the animal represents the time when replenishment of the lipid stores of the brown fat was beginning. Fig-

ure 54 thus shows the presence of tritium in the lipid droplet. Other fields showed the label in other places—within the mitochondria, in the capillary walls, etc.

CONCLUSION

In the euthermic or hibernating ground squirrel, the lipid droplets are large and are virtually surrounded by mitochondria. Upon arousal, much of the lipid is rapidly metabolized to provide heat; many of the droplets are entirely consumed, but the decrease in size is not uniform. A study of the process of replenishment of the tissue lipids after the peak of arousal activity should prove instructive.

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THE ULTRAVIOLET SPECTRA OF NUCLEOSIDES AND DNA IN D_2O

Lyle G. Bunville and Sandra J. Schwalbe

PURPOSE AND METHODS

The investigation of aqueous solvent effects on the spectra and physical properties of DNA and DNA

TABLE 7. HYDROGEN BONDING IN AQUEOUS NUCLEOSIDE SOLUTIONS. ISOTOPE EFFECT ON THE STRENGTH OF SOLUTE-SOLVENT HYDROGEN BOND

Nucleoside	$\Delta\lambda$, Å	$-\Delta\epsilon$	ΔE , kcal/mole
dA	6.2	620	0.24
dG	7.3	370	0.25
dC	5.5	290	0.19
T	3.2	140	0.11

components reported earlier⁽¹⁾ has been extended to include the spectra of the deoxyribo- and ribonucleosides in D_2O . In each instance difference spectra have been determined directly as $\Delta\epsilon = \epsilon(n_d) - \epsilon(n_d = 0)$ as a function of n_d , atom fraction of deuterium in the solvent.

PROGRESS REPORT

The various nucleosides that have been studied are adenosine(A), deoxyadenosine(dA), guanosine(G), deoxyguanosine(dG), deoxycytidine(dC), and thymidine(T). The results for the deoxynucleosides are summarized in Table 7. In Figure 55 the long-wave-

FIG. 55.—Maximum extinction coefficient change for the deoxynucleosides in D₂O (compared to H₂O) as a function of the number of aza nitrogens (N_{aza}). Numbers in parentheses are wavelengths (mμ) of maximum change.

length maximum change in extinction coefficient is shown as function of the number of aza nitrogens (N_{aza}) of the purine or pyrimidine base. For each nucleoside the change in extinction coefficient is linear with the atom fraction of deuterium in the solvent.

CONCLUSIONS

The long-wavelength decrease in the extinction coefficient of the various nucleosides has been interpreted as arising from increased stabilization of the ground state of lone-pair electrons on the purine or pyrimidine residues of the nucleosides through the formation of a stronger hydrogen bond with solvent D₂O than with H₂O. From the magnitude of the decrease in extinction coefficient, the increase in the

strength of the hydrogen bond has been estimated as⁽²⁾

$$\Delta E = -285.7 \frac{\Delta E}{(d\epsilon/d\lambda)\lambda^2} \text{ kcal/mole}$$

where, at the wavelength λÅ, Δε is the extinction coefficient difference, and (dε/dλ) the first derivative of the absorption spectrum. The estimated differences in the strength of the hydrogen bond for the various deoxynucleosides are given in Table 7.

That spectral changes in D₂O solutions of the nucleosides do not arise solely from solvent hydrogen bonding at the aza nitrogens is seen in the small change in extinction coefficient for thymidine, which has no aza nitrogens (Figure 55).

Although the results presented here indicate a qualitative correlation of extinction coefficient change with the number of sites available for the formation of a particular type of hydrogen bond with the solvent, lack of information as to the number of sites available for such hydrogen bond formation in helical DNA or the effect of solvent exchange on the conformation of helical DNA preclude a detailed comparison of the results for the nucleosides with those for DNA. The change in extinction coefficient observed for DNA (native) is approximately 25% of that expected for a mixture of noninteracting nucleosides.

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THE CONTROL OF MACROMOLECULAR SYNTHESIS IN BACTERIA

Richard E. Ecker and Gloria Kokaisl

PURPOSE AND METHODS

In cultures of bacteria, the rate of protein synthesis in balanced growth is directly proportional to the cellular ribosome content.⁽¹⁾ This holds for a wide range of growth rates, established under a variety of conditions and, thus, has suggested that individual ribosomes operate at all times with essentially constant efficiency. However, cultures of growing bacteria contain a distribution of cells within the cell division cycle. Thus, even in balanced growth, a bacterial culture represents a very heterogeneous popula-

tion in terms of cell size and cell age (time since division), and data obtained using such cultures represent only averages over entire populations. It is conceivable, then, that the data obtained using bacterial populations could accurately indicate that ribosomes in the average cell function with the same efficiency, irrespective of the growth rate; at the same time, ribosomes within individual cells could actually change markedly in efficiency through the cell division cycle. The present investigation was initiated to distinguish between these two possibilities.

We have used a microautoradiographic technique to observe the distribution of synthetic rates and macromolecular content in individual cells as a function of cell size. Cells in balanced growth were exposed to tritium-labeled leucine (protein precursor) for a very short period (pulse), then killed with trichloroacetic acid, and the unincorporated leucine was extracted. The extracted cells were then transferred to microscope slides, coated with photographic emulsion, and stored for exposure. When the time of exposure was sufficient to give an average of about 3 or 4 grains/cell, slides were developed, and the cells were stained through the emulsion. With an ocular micrometer, each of the cells in a number of randomly-selected fields was sized, and the number of grains associated with each cell was recorded. Since the incorporation time was very short relative to the generation time, grains/cell becomes a relative measure of the rate of protein synthesis in each individual cell at that time.

In addition, to determine RNA and protein content as a function of cell size, the cultures were labeled for 4 generations (steady-state labeled) with uracil or leucine and then treated as above. In this case, grains/cell becomes a relative measure of total content of either RNA or protein in individual cells. Finally, to determine the fraction of the total RNA that is ribosomal, cells which had been steady-state labeled with uracil were lysed, and the cell constituents were separated on a sucrose gradient by high-speed centrifugation. Incorporated activity in the various RNA-containing constituents was then determined.

PROGRESS REPORT

Cell division in bacteria is not a precisely timed event. As a result, in the cell size distribution of a bacterial culture, about 10% of the total cells are larger than the expected size at division, and about 10% are smaller than half the expected division size. This means that, although one would expect cells to vary no more than twofold in size during the division cycle, they actually vary almost fourfold.

We have found that, over this entire range of cell sizes, the rate of protein synthesis in a cell is proportional to the size of the cell, and that the rate extrapolates to zero at zero cell size. In mathematical terms this says

$$dP/dt = k_1L, \quad (1)$$

where dP/dt is the rate of protein synthesis, L is the cell length (in these cultures growth is almost exclusively longitudinal) and k_1 is a constant. Similarly, the cellular content of both protein and total RNA is directly proportional to cell size. In steady-state labeled cultures growing at moderate rates, ribosomes account for more than 80% of all uracil incorporated into RNA, and this proportion varies only slightly with growth rate. Therefore, most of the RNA made under these conditions becomes part of ribosomes. So we can now introduce the relationship

$$R = k_2L, \quad (2)$$

where R is the cellular ribosome content and k_2 is another constant. Combining equations (1) and (2), we obtain

$$dP/dt = k_1/k_2R \text{ or } dP/dt = KR. \quad (3)$$

CONCLUSIONS

The rate of protein synthesis in individual cells is proportional to the cellular content of ribosomes. Thus, we can say that ribosomes function at constant efficiency within individual cells during the cell division cycle. However, we still cannot say with assurance that individual ribosomes function with constant efficiency, for even with growing individual cells there could be a population of ribosomes having a distribution of efficiencies, the mean of which is measured during our experiments.

The cellular content of both protein and RNA increase linearly with cell size. These are the major macromolecular constituents of the cell. If we assume, therefore, that protein content reflects a valid measure of cell size, we can change equation (1) slightly to read

$$dL/dt = k_3L \text{ or } L = L_0e^{k_3t}, \quad (4)$$

where L_0 is the cell size at birth, that is, immediately after division. This predicts a size-age relationship, then, and suggests that the growth of individual cells is exponential with time.

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SPECTROPHOTOMETRIC APPARATUS FOR THE AUTOMATIC DETECTION OF PEPTIDES AND PROTEINS

Allen B. Edmundson, William R. Cole, Florence A. Sheber, Nancy K. Hutson, Kathryn R. Ely, and J. William Harrison

PURPOSE AND METHODS

In the elucidation of the amino acid sequence of sperm whale myoglobin,^(1, 2) all peptides liberated by chemical or enzymatic techniques were detected by a photometric ninhydrin method⁽³⁾ after alkaline hydrolysis⁽⁴⁾ of small aliquots. For reasons given in the previous Annual Report,⁽⁵⁾ it was desirable to supplement these colorimetric analyses with spectrophotometric methods for the automatic detection of peptides and proteins. The procedures we developed were generally based on the absorption of the peptide bonds at 230 m μ or of the tryptophanyl and tyrosinyl side-chains at 280 m μ , although other wavelengths throughout the ultraviolet and visible regions were selected in special cases (e.g., 258 m μ for phenylalanyl side-chains or 409 m μ for the Soret band of myoglobin). A Gilford Model 2000 Multiple Sample Absorbance Recorder was used for the detection, and the readings were recorded directly in absorbance units. This recording spectrophotometer is accurate, sensitive, and stable (see Progress Report) and can be employed without modification. However, the development of the remainder of the analytical system required considerable research time. In the course of the experiments we devised several chromatographic aids of general applicability, and these will also be briefly described here.

The essential features of the apparatus are indicated by the flow diagram in Figure 56. The elution system, shown at the top of the figure, may involve a single liquid or a gradient of the logarithmic, linear, or variable types. The eluents most commonly used in our laboratory are single liquids or simple logarithmic gradients. For the latter, we have accordingly constructed tables with which we can predict the concentration or pH of the eluents emerging from the gradient-making devices. Miss Jeanne Pagels has recently written a computer program for constructing these tables more rapidly.

The entry of the eluents into the analytical train is regulated by a series of solenoid-controlled valves, designed by William F. Eisler, Jr. In Figure 56 the valves are open to the logarithmic gradient.

The eluent is pumped through a jacketed column by a piston or peristaltic pump equipped with a shut-down timer to terminate the chromatography automatically at night or on weekends. Effluent from the column is passed into the spectrophotometer and through silica flow-cells. The commercially available cells are spe-

Fig. 56.—Flow diagram for the automatic detection of proteins and peptides with a spectrophotometric apparatus.

cially modified with Teflon pressure-fittings to prevent leakage. While simple in design, each T-shaped fitting was carefully machined to have the diameter of the sealing arm 0.007" greater than the diameter of the port into which it was to be forced. The fittings have not leaked under operating conditions in which the pressure at the tops of the columns has been as high as 300 psi. A standard microcuvette holder was altered to accommodate the cells and the fittings.

The stream emerging from the flow cells is passed into a fraction collector. To correlate the spectrophotometric readings with the fraction numbers, an event marker on the recorder is automatically activated during each change of fractions.

For temperature control, which is essential in the fractionation of proteins and peptides, water from thermostatically controlled baths can be circulated through the jacket of the column and the housing of the flow-cells. When the eluent and effluent must be kept cold, as in the purification of labile proteins, the fraction collector can be refrigerated, and the eluent can be immersed in a refrigerated water bath and stirred with a specially designed immersible stirrer. This instrument has also been successfully employed over the temperature range of 0 to 60° C in other applications requiring a magnetic stirrer.

To reduce mixing, the flow lines from the column to the collector consist of short segments of narrow #22

FIG. 57.—Photographs of modified carts holding components of the spectrophotometric apparatus

AWG heavy-walled Teflon tubing. For convenience in assembly and dismantling, the lines are joined through 16-gauge clear polyvinyl sleeving.

Inexpensive carts were modified with plywood platforms to make the equipment as mobile as possible without loss of stability. Photographs of the equipment placed on the carts are presented in Figure 57. On the smaller cart (see left panel of Figure 57) are a fraction collector, a piston pump, and a water bath. The heavier Gilford spectrophotometer and its desirable accessories (see right panel of Figure 57) are placed on a larger cart made by bridging two smaller carts with two layers of $\frac{3}{4}$ " plywood secured to the inverted top trays and with one layer of plywood bolted to the bottom trays of the carts. The carts were modified and assembled by Walter J. Sztuczko, Elmer C. Braasch, and Walter A. Sak.

For manual spectrophotometric determinations, the analytical system is equipped with a digital readout and an instrument (Transferator) for the automatic transfer of samples to and from the cuvettes. A transfer adapter was designed to improve the efficiency of the latter instrument. Improvements were also made

in other instruments used in chromatographic procedures, such as rotary evaporators and automatic pipetting machines.

We shall be very happy to supply details on the design and construction of the flow-cell pressure fittings, immersible stirrer, chromatographic carts, transfer adapter, and modifications of the rotary evaporators and automatic pipetting machines.

To illustrate the use of the analytical system, a mixture of peptides from a chymotryptic hydrolysate of a Bence-Jones λ -chain (Hulette-Way)⁽⁶⁾ was subjected to permeation chromatography on a 2.5×180 -cm column of Sephadex G-25, a modified dextran gel. The peptides were automatically detected at two wavelengths, 230 and 280 m μ . The accessories required for the two measurements with the spectrophotometer were a dual wavelength selector and an automatic blank compensator.

PROGRESS REPORT

The analytical system has proved to be highly adaptable and sensitive in the detection of proteins and peptides. For example, peptides containing two or

more amino acid residues have been detected at levels below five nanomoles. The high sensitivity is partly attributable to the fact that the entire sample is monitored. Furthermore, the scale on the recorder can be expanded to register only 0.1 absorbance unit at maximum deflection. For higher concentrations of sample the scale can be adjusted to values as high as 3.0 absorbance units without loss of accuracy. This degree of accuracy is possible because the output voltage of the photomultiplier in the spectrophotometer is proportional to the absorbance throughout the range of 0 to 3 absorbance units. We confirmed the linearity of this relationship for all eluents employed in our procedures.

The nondestructive nature of the analytical system and the avoidance of stream-dividing permit complete recovery of the samples. This recovery is particularly important for samples in limited quantities and for multistage fractionation procedures. Moreover, the separated components are available for other types of analyses or for manual reexamination if technical difficulties are encountered. The latter usually are minor and most commonly involve the presence of air in the flow-cells.

While additions and improvements continue to be made, the prototypes of the chromatographic aids described in this article have proved reliable for test periods varying from three months to three years. Among these aids, the chromatographic carts have elicited the most interest in laboratories outside Argonne. They are inexpensive, easily constructed, and can be wheeled by one person through openings as narrow as 30 in. The performance data, range of applicability, and advantages and limitations of the remaining aids will not be discussed here, but this information can be obtained from the authors.

The elution pattern for the partial fractionation of the group of chymotryptic peptides is shown in Figure 58. For clarity only the pertinent part of the graph is presented, and the experimental points on the actual tracing are connected with solid lines. The fraction numbers are indicated by marker lines on the abscissa. To accentuate the absorbance values at 280 $m\mu$, full scale on the recorder was set at only 0.5 absorbance unit. Between each reading the absorbance of the blank was automatically readjusted to 0 and printed out for each wavelength. The print-out indicates that the mixture was separated into four components on the

FIG. 58.—Effluent curve for the spectrophotometric detection of chymotryptic peptides from a Bence-Jones protein (Hulette-Way).

column. It can be concluded from the absorption at 280 $m\mu$ that peptides in each of the four bands contain aromatic residues of tyrosine and/or tryptophan, in accord with the known specificity of chymotrypsin. In addition to the applications with gels, spectrophotometric techniques have been used with other supporting media, such as Amberlite CG-50, a weak cation exchanger, BioRad AG 50-X2, a strong cation exchanger, and BioRad AG 1-X2, a strong anion exchanger. These applications have been successful in all cases except those in which eluents strongly absorbing in the ultra-violet are essential for acceptable resolution on the chromatographic columns.

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STUDIES ON NUCLEAR PROTEINS

Ambrose D. Barton

PURPOSE AND METHODS

These studies on nuclear proteins stem from a long-term interest in the organization and functions of the cell nucleus. Much of the previous work on this project has dealt with a class of soluble proteins that are retained when rat liver cell nuclei are isolated in sucrose media and are liberated when the isolated nuclei are extracted with an ionic medium, such as isotonic saline. These saline-soluble proteins of the cell nucleus are relatively basic compared with the soluble proteins of the cytoplasm, although not so basic as histones. They are retained in the nucleus by ion binding, and their behavior in moving-boundary electrophoresis changes with changes in the physiological status of the animal.⁽¹⁾ More recently, these studies have been extended to include the investigation of other types of nuclear protein under conditions in which the metabolic condition of the animal is altered. For example, we found that the crude histone fraction obtained from isolated, washed nuclei by acid extraction was increased substantially during the nuclear enlargement that is produced by administration of thioacetamide.^(2, 3) It has been found that this increase occurs in an anomalous type of protein that resembles histone but having a lower content of basic amino acids,⁽⁴⁾ a higher content of acidic amino acids and a higher proportion of the distal carboxyl groups present in the form of neutral amide groups. The results indicated that these anomalous proteins must be present in the chromatin and are not merely constituents of the nucleolus.

PROGRESS REPORT

In our previous studies, the chromatin fraction was obtained by homogenizing the washed nuclei in 2 *N* NaCl, and removing the nucleoli by centrifugation, leaving the chromatin fraction in the supernatant. Unfortunately, while this fraction contains the constituents of the chromatin they have been largely dissociated and much of the original chromatin organization has been destroyed. More recently, we have worked out procedures for disrupting the nuclear structure with sonic oscillation, so that the nucleoli can be eliminated by centrifugation. This procedure has the advantage that it can be carried out while the more readily extractable protein fractions are still attached to the chromatin. In the resulting chromatin suspension, approximately four fifths of the DNA is in fragments that sediment fairly rapidly, and the rest is in fragments that sediment much more slowly. However, the latter include a larger proportion of the chromatin

RNA and protein. These suspensions of chromatin fragments are being studied by means of density gradient centrifugation, the objective being to obtain more definite location of these nuclear constituents in particular nuclear structures.

As reported earlier,⁽⁴⁾ we found that our protein fractions differed somewhat in amino acid composition from the whole protein fraction from ribosomes. More recently, Atsmon et al.⁽⁵⁾ reported on the ribosomal proteins most resistant to extraction by high concentrations of LiCl and urea. In their lower content of basic amino acids, their higher content of acidic amino acids, and higher number of distal carboxyl groups present in the neutral amide form, these ribosomal proteins correspond almost exactly with some of our later chromatographic fractions. Thus, some of our anomalous proteins may be associated with ribosomes, or with ribosome precursor particles in the nucleus. However, much of the chromatin RNA is not of ribosome composition, and presumably it has basic protein associated with it also. It is expected that the present experiments on density gradient fractionation of chromatin fragments will provide a means for exploring these possibilities.

Much of the protein in our crude histone fraction (containing histones and our anomalous proteins), is highly associated. It is almost completely excluded from Sephadex G75, and a considerable part of it is scarcely able to enter the gel in polyacrylamide gel electrophoresis. This association is not readily reversed by urea, even in concentrations as high as 8 *M*. These anomalous proteins are soluble in dilute salt solutions, but aggregate and precipitate when the salt concentration is increased, i.e., when the charged groups are screened and the electrostatic repulsion is reduced. They redissolve readily when the salt concentration is reduced by dialysis. Their tendency to associate is greatly reduced when the free amino groups are converted to carboxyl groups by treatment with maleic anhydride, which reduces the possibility of salt bonds and greatly increases the electrostatic repulsion between the charged groups since they are now essentially all of the same sign. After this treatment it is evident that they are actually proteins of low molecular weight, as indicated by their behavior in gel filtration in Sephadex G75, and their rapid migration in gel electrophoresis. These anomalous proteins have a relatively high content of the aliphatic amino acids, isoleucine, leucine, and valine, (18 to 25 residues per hundred).

CONCLUSIONS

All of these results suggest that the association and aggregation of these anomalous proteins is due primarily to hydrophobic bonding. Thus, these relatively small protein molecules are able to associate to form stable components of nuclear structures by a type of bonding that is readily reversible under suitable conditions. This ready reversibility may be of importance for their functioning in the chromatin.

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ULTRAVIOLET MICROSCOPY OF PURINES AND AMINO ACIDS IN THE VACUOLE OF CANDIDA ALBICANS*

George Svihla and Edward Balish

Ultraviolet microscopy was used to study the capacity of yeast (ATCC 10231 and 10261) and filamentous (ATCC 10259) strains of *Candida albicans* to accumulate ultraviolet-absorbing materials from a medium supplemented with purines, pyrimidines, amino acids, or related compounds as the main nitrogen source. All strains accumulated ultraviolet-absorbing compounds when adenine, adenosine, isoguanine, xanthine, or uric acid was supplied as a nitrogen source, but they did not accumulate ultraviolet-absorbing compounds when pyrimidines were supplied. The fila-

mentous strain accumulated ultraviolet-absorbing material from medium supplemented with hypoxanthine, but the yeast strains did not. In contrast, the yeast strains accumulated more ultraviolet-absorbing material than did the filamentous strain when guanine was the nitrogen source. Yeast strain 10231 not only accumulated ultraviolet-absorbing material from tyrosine supplemented medium, but it became filamentous in form as well. Yeast strain 10261 and the filamentous strain 10259 were not appreciably affected by tyrosine. The two yeast strains accumulated more lipid than the filamentous strain when they were incubated in the nitrogen deficient medium.

* Abstract of a paper to be submitted for publication.

Enzymology

THE DISTRIBUTION OF MITOCHONDRIAL ENZYMES IN RAT LIVER AFTER VARIOUS TREATMENTS, IN LIVER FROM OTHER SPECIES, AND IN RAT HEART

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PURPOSE AND METHODS

Parenchymal cells in the peripheral region of the liver lobule have been shown to differ from those in the central zone. Of particular interest to us were the apparent differences in the mitochondria from these two areas of the lobule. From electronmicrographs, Novikoff and Shin⁽¹⁾ concluded that mitochondria in the pericentral cells were smaller than those in the peripheral cells. Bahr and Zeitler⁽²⁾ found two populations

of mitochondria, one round, the other oblong, each with its own weight distribution. A number of studies, using histochemical techniques⁽³⁾ or microdissection,⁽⁴⁾ have shown differences in the activity of several enzymes across the profile of the lobule. Using the increased sensitivity of the zonal gradient centrifuge, we have distributed enriched preparations of rat liver mitochondria according to size, and have measured the activity of twelve enzymes in the fractions obtained.

The midpoint of each distribution was calculated by three independent methods: (1) from the weighted probability transform (probit), (2) from the ratio of total activity of one enzyme to that of another or to total protein in successive fractions,⁽⁵⁾ and (3) by fitting a normal curve to the frequency distribution by the method of least squares.⁽⁶⁾ A consistent and significant displacement of the midpoints of the distribution of many enzymes from each other, or from total protein, was obtained.⁽⁷⁾ This midpoint was expressed in terms of the mean particle diameter of the mitochondria found at that point. The separation, or differences in midpoints, between enzymes or enzyme and total protein will be expressed as a percent of the mean particle diameter of glutamate dehydrogenase, a useful reference enzyme.⁽⁷⁾

PROGRESS REPORT

SD/Anl [Anl 66] rats were treated to induce changes in their liver cell population and in the size, integrity, and enzyme complement of liver mitochondria in order to determine whether these changes are accompanied by an alteration in the distribution of mitochondrial enzymes examined previously.⁽⁷⁾ Preliminary consideration of the data obtained from a large number of experiments indicated that while the size of the mitochondria can be altered markedly, no very striking changes in the distribution of their enzymes was produced.

For example, subtotal hepatectomy triggers rapid cell division in an otherwise quiescent tissue. Within a few hours, the rate of DNA synthesis and cell division increases, and this rate continues until the liver has been restored to its original mass. Mitochondrial formation in these cells lags somewhat behind cell division.⁽⁸⁾ We have observed that the mean particle diameter of the mitochondria, as reflected by the midpoint of the total protein distribution, shrinks from 0.94μ to 0.83μ by day 3 post-hepatectomy and returns to the control value by day 13. During this period, however, the distribution of eight enzymes (ornithine aminotransferase, cytochrome oxidase, succinate dehydrogenase, 3-hydroxybutyrate dehydrogenase, malate dehydrogenase, glutamate dehydrogenase, aspartate aminotransferase, and ornithine transcarbamoylase) does not change significantly in relation either to each other or to the total protein.

When rats are depleted of essential fatty acids or are treated with the corticosteroid, prednisolone, the mean particle diameter of the mitochondria increased as expected^(9, 10) to 0.99 – 1.15μ in response to essential fatty acid depletion and to 1.11 – 1.16μ in response to treatment with hormone. The midpoints of the distri-

bution of cytochrome oxidase and of ornithine aminotransferase were not affected as greatly as midpoints of the other enzymes; hence, their separation from the other enzymes was slightly increased.

We were concerned that the apparent smaller mean particle diameter of mitochondria (based on sedimentation characteristics) 3 days post-hepatectomy might be the result of an altered density of mitochondria from these livers which had become fatty. The administration of prednisolone, however, also produced fatty livers, but their mitochondria were larger than normal, in agreement with the findings of others.⁽¹⁰⁾ Furthermore, mitochondria from hepatectomized animals were also sedimented in an equilibrium density gradient (10–80% sucrose). An equilibrium density of 1.22 g/ml was found for the mitochondria from both the fatty livers and the control animals. Therefore, the degree of fattiness of a liver apparently has no effect on the sedimentation of its mitochondria. This response may not hold true for mitochondria prepared from other tissues.⁽¹¹⁾

While the level of alanine aminotransferase activity doubled in the hepatic mitochondria of rats treated with prednisolone, the midpoint of its distribution, with respect to that of glutamate dehydrogenase, was virtually unchanged: 99.2–99.3% of the value for glutamate dehydrogenase vs. 100.2% in control animals. The mean particle diameter of mitochondria in rats fed diets high in protein also increased. The specific activity of alanine aminotransferase doubled, but the distribution again remained unchanged: 99.4–99.7% of the midpoint of the distribution of glutamate dehydrogenase. Ornithine aminotransferase activity also increased sharply in these rats. Here, the midpoint was 97.5% of the value for glutamate dehydrogenase as compared to 95.5% in control animals.

The administration of CCl_4 causes necrotic areas to develop in the central regions of the liver lobule. The destruction of these cells and their smaller mitochondria would be expected to produce a shift in the mean particle diameters of the distributions to a larger value. This did not occur; all mitochondria apparently were affected, and the mean particle diameter of the midpoints decreased for all of the enzymes. Ornithine aminotransferase activity, however, which may be uniquely associated with small mitochondria,⁽⁷⁾ almost disappears from mitochondria prepared from animals given CCl_4 .

X irradiation has been claimed by many workers to have an effect on liver mitochondria; however, few of these observations have been confirmed by careful experiments done here. In a single experiment, we could find no significant deviation from normal in either the

mean particle diameter or in enzyme distributions of mitochondria prepared 4 hr after whole-body irradiation with 1000 R of 250-kVp X irradiation.

Liver mitochondria were prepared from a number of other species and sedimented through a sucrose gradient. Results obtained with Chinese hamster, ground squirrel and guinea pig liver mitochondria were similar to those seen with rat liver, although the mean particle diameter of the midpoints were all 10 to 20% larger. Ornithine aminotransferase was essentially absent from guinea pig liver mitochondria. Mouse liver mitochondria gave excellent "normal" distributions and were more interesting, however. In three experiments, the mean particle diameter of the midpoint of the distribution of ornithine aminotransferase was 83.6, 87.8, and 88.7% of that of glutamate dehydrogenase. This is a remarkable degree of separation. The distributions of cytochrome oxidase and 3-hydroxybutyrate and succinate dehydrogenases were also quite displaced, while those of malate and glutamate dehydrogenase and aspartate aminotransferase were nearly identical.

We have proposed that the differences in the distribution of these enzymes in liver mitochondria reflect differences in the metabolic capacity of centrilobular cells containing small mitochondria and peripheral cells containing larger mitochondria. A possible test of this hypothesis would be a study of the distribution of enzymes in mitochondria from some source more homogeneous than liver. Using rat heart mitochondria prepared according to the method of Tyler and Gonze,⁽¹²⁾ we found no significant separation of the midpoints of the distributions of glutamate, isocitrate, malate, and 3-hydroxybutyrate dehydrogenases and aspartate aminotransferase. The distributions of succinate dehydrogenase and cytochrome oxidase differ from each other, however, and from the other enzymes such as those in mitochondria from liver. Unfortunately, ornithine aminotransferase does not occur in rat heart mitochondria. Therefore, these results are equivocal, and more work will be done with mitochondria from heart and other sources to determine the significance of the differences we find in liver.

CONCLUSIONS

A number of conditions were examined which are known to affect the size of liver mitochondria. Although several altered the mean particle diameter of the mitochondria as expected, only minor changes in the distribution of the enzymes, relative to one another, were seen. Even when CCl₄ was given, where some fragmentation of the mitochondria was evident, the relative distribution of the enzymes was essentially unchanged.

It has been suggested that mitochondria arise by the growth and division of existing mitochondria. If this is true, smaller mitochondria would represent young mitochondria. Such younger and smaller mitochondria would be expected to contain increased amounts of those enzymes responsive to a gluconeogenic stimulus shortly after its initiation. At these early times, therefore, one might expect a shift in the midpoints of the distributions of the susceptible enzymes to a smaller mean particle diameter. This shift was not seen in the distribution of alanine and ornithine aminotransferase during a period when their activity was increasing rapidly. Indeed, the mean particle diameter of the distribution of ornithine aminotransferase *increased* relative to the other enzymes, suggesting that the level of activity now increased or increased more rapidly in larger mitochondria than it did in smaller ones. These results suggest, therefore, that all mitochondria are in a dynamic state; alteration of their enzyme complements is independent of mitochondrial genesis.

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LABELLED S-ADENOSYLMETHIONINE

Fritz Schlenk

PURPOSE AND METHODS

The rapidly increasing interest in the biological sulfonium compound S-adenosylmethionine has made the biosynthesis of labeled modifications of this compound desirable. The most familiar function of this compound in cells is that of a methyl donor in biological transmethylation. Accordingly, the methyl-

FIG. 59.—Some features of methionine metabolism in yeast

FIG. 60.—The effect of unlabeled L-methionine supplement (O—O) in the culture medium of the yeast *Candida utilis* on the incorporation of ^{35}S from $^{35}\text{SO}_4^{--}$ into S-adenosylmethionine. The theoretical values for the dilution of $^{35}\text{SO}_4^{--}$ in the medium initially (0.6 $\mu\text{moles/ml}$) by ^{35}S from the supplement are represented by X—X. The specific radioactivity of $^{35}\text{SO}_4^{--}$ in the culture medium was 2.9×10^6 cpm/ μmole . Experimental details of the production of ^{35}S -adenosylmethionine from sulfate are given in an earlier report.⁽⁴⁾

labeled material is of particular importance, while several other sites of labeling have been essential for studies of additional functions of this sulfonium compound.

Biosynthesis of S-adenosylmethionine by yeast from labeled precursors, as elaborated in this laboratory,⁽¹⁾ proved to be the only practical method for securing the material in quantity. These procedures have been improved in various ways.^(2, 3) Particular attention has been given to the radiopurity of S-adenosylmethionine.

The most important types of radioactive S-adenosylmethionine are obtained from methionine which is labeled in the requisite position. In yeast cultures, methionine is assimilated from the medium and reacts with adenosine triphosphate (ATP) in the cells:

This, however, is not the only route of methionine metabolism; several other important reactions are represented by Figure 59. Various degradation products of labeled methionine may find their way into the cellular metabolic pool and thence may participate in a nonspecific way in the endogenous biosynthesis of methionine and adenosine triphosphate. This would lead to undesired, nonspecific labeling of S-adenosylmethionine.

PROGRESS REPORT

The level of radioimpurity present in biosynthetic preparations of S-adenosylmethionine was examined in some detail. Obviously, the occurrence of stray labeling depends on the level of the endogenous synthesis of methionine in the presence of extraneously supplied methionine. A repressing effect of the latter would be beneficial. This was tested by assay of the incorporation of ^{35}S from $^{35}\text{SO}_4^{--}$ in the medium into S-adenosylmethionine (Figure 60). It is apparent that external methionine has a strong repressing effect on the endogenous synthesis of this amino acid. This restricts randomization of the label by endogenous methionine

and S-adenosylmethionine biosynthesis from constituents of the metabolic pool.

In a more direct approach, samples of labeled S-adenosylmethionine obtained from L-methionine- $^{14}\text{CH}_3$ were hydrolyzed to adenine and homoserine. These products were purified carefully and analyzed for stray isotope. The radioactivity found in several samples of adenine averaged less than 0.5%, and that of homoserine less than 0.2% of the specific radioactivity of the parent material. Supplementation of the culture medium with adenine or adenosine in addition to labeled methionine did not reduce the level of undesired isotope in the adenine of the resulting S-adenosylmethionine; apparently, the endogenous purine synthesis was not significantly suppressed.

CONCLUSION

The level of radioimpurity in the adenine and homoserine moiety of S-adenosylmethionine- $^{14}\text{CH}_3$ is too low to warrant concern in enzymatic experiments with currently available analytical techniques. Some decomposition products of S-adenosylmethionine are present in most preparations at the outset and are

formed also during enzymatic experimentation with solutions near neutrality; their level exceeds that of the radioimpurities observed at present by an order of magnitude. Moreover, the analytical methods available for transmethylation studies are not yet accurate within a fraction of a percent.

The present study of radioimpurities in S-adenosylmethionine suggests analytical surveys in other instances of microbial biosynthesis, in which an excess of labeled precursor is used for the production of a complex product.

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ENZYME REGULATION IN THE PATHWAY OF METHIONINE BIOSYNTHESIS. MOLECULAR CONVERSION OF ADENOSYLHOMOCYSTEINE TO ADENOSYLMETHIONINE

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PURPOSE AND METHODS

Scheme 1 illustrates the terminal steps in the biosynthesis of methionine, adenosylmethionine, adenosylhomocysteine, and their respective degradation products. A review of our previous studies, in which all of these enzymatic reactions were described, has been published.⁽¹⁾ Current efforts are directed toward studies of the interrelationship of these pathways, with emphasis on the control mechanisms involved. One of the critical problems of relevance to this scheme involves the metabolic fate of adenosylhomocysteine. The possibility of a direct regeneration of adenosyl-

homocysteine (AH) to adenosylmethionine (AM) is of great interest, since AH is a product of numerous cellular transmethylation reactions.

PROGRESS REPORT

Our results have shown that the addition of 1 $\mu\text{mole/ml}$ of adenosylhomocysteine to a minimal medium results in the formation of up to 2 μmoles of AM per gram of cells of *Candida utilis*. Since this is approximately twice the amount of AM that is found in the unsupplemented cultures, it was considered important to determine whether AH represses the endogenous formation of AM. It may be seen (Table 8) that the presence of methionine represses endogenous AM synthesis virtually completely, and AH represses endogenous synthesis by about 15 to 20%.

AH labeled in various positions was used to determine the mechanism of AM synthesis from AH. The results are in Figure 61. The curves at the right are plotted on the basis of complete repression of endogenous AM synthesis. In either case, the presence of unlabeled adenosine dilutes the specific activity of

TABLE 8. FORMATION OF ADENOSYLMETHIONINE (AM) FROM ADENOSYLMETHIONINE (AH)

Sulfur supplement in medium (0.8 μ mole/ml)	Wet weight, mg	AM formed, μ moles	Incorporation ^(a) of ^{35}S , %
$^{35}\text{SO}_4$	532	0.530	93.0
$^{35}\text{SO}_4$	527	0.537	92.8
$^{35}\text{SO}_4$ + Methionine ^(b)	482	2.818	0.18
$^{35}\text{SO}_4$ + Methionine ^(b)	488	2.840	0.19
$^{35}\text{SO}_4$ + Adenosylhomocysteine ^(b)	537	0.521	75.1
$^{35}\text{SO}_4$ + Adenosylhomocysteine ^(b)	542	0.510	72.9

(a) $100 \times$ specific activity of isolated AM/specific activity of $^{35}\text{SO}_4$.

(b) Concentration was 2.0 μ moles/ml.

FIG. 61.—Percent incorporation of label from A*H or AH* into AM isolated from *Candida utilis* grown for 44 hr with various concentrations of adenosine.

AM, when AH-adenosine-8- ^{14}C is used as the label, in proportion to the amount of adenosine added to the medium. When AH- $^{14}\text{COOH}$ is used in the presence of adenosine, there is no dilution of the specific activity of the resultant AM. Similar results were obtained when cold adenine was added to the growth medium with the labeled AH. These results suggest that AH is decomposed and resynthesized to AM. It was considered possible that the dilution of the specific activity of AM-adenosine-8- ^{14}C was due to an exchange reaction between AH and adenosine and a subsequent methylation of AH directly to AM. If this were the

$^{100} \times$ specific activity of Adenosylmethionine/specific activity of Adenosylhomocysteine

FIG. 62.—Percent incorporation of ^{14}C or ^3H into AM isolated from *C. utilis* grown for various periods of time with AH-adenosine-8- ^{14}C (A*H) with and without adenosine- ^3H .

FIG. 63.—Percent incorporation of label into AM isolated from *C. utilis* grown for various periods of time with AH- $^{14}\text{COOH}$ with and without unlabeled homocysteine.

mechanism, then the specific activity of AM should decrease with time when cold adenosine and AH-adenosine-8- ^{14}C are in the medium. The results of an experiment in which the specific activity of AM was measured at various times are shown in Figure 62. It may be seen that the specific activity of the resultant AM increased with time when A*H alone was used or A*H + adenosine was used. Similarly, when AH- $^{14}\text{COOH}$ was in the medium, the specific activity of the AM formed increased with time in either the presence or absence of cold homocysteine (Figure 63).

CONCLUSIONS

Under the conditions of these experiments, the results are consistent with the hypothesis that AH stimulates the production of AM via decomposition of the molecule and not via direct addition of a methyl group to AH.

METHIONINE BIOSYNTHESIS IN CANDIDA ALBICANS

Edward Balish and Stanley K. Shapiro

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PURPOSE AND METHODS

Methionine is not only required for protein synthesis, it is also involved in sulfur metabolism as well as in numerous methyltransferase reactions where it is part of the ubiquitous methyl donor, adenosylmethionine.⁽¹⁾ Methionine has also been implicated in the control of cellular form in the yeast, *Trigonopsis variabilis*,⁽²⁾ and in the morphogenesis of *Candida albicans*.⁽³⁾ Recent studies on the mechanism of methionine biosynthesis in microorganisms have been confined to *Saccharomyces cerevisiae*, *Escherichia coli*, and *Aerobacter aerogenes*.⁽¹⁾ Because of the suggested unique role for methionine in *C. albicans*, an investigation of methionine metabolism in this organism was undertaken. Two yeast phase strains and a mycelial phase strain were used in these experiments.

PROGRESS REPORT

The results shown in Table 9 demonstrate that cell-free extracts of the yeast phase cultures (ATCC 10231

TABLE 9. STIMULATION OF HOMOCYSTEINE METHYLTRANSFERASE ACTIVITY OF *C. albicans* CULTURED IN THE PRESENCE OF METHIONINE

Enzyme source	Supplement in medium	Methionine/mg protein/hr, μ mole	
		Methyl donor	
		Adenosylmethionine	Methylmethionine
<i>C. albicans</i> 10231	—	44	122
<i>C. albicans</i> 10231	Methionine	77	240
<i>C. albicans</i> 10261	—	155	336
<i>C. albicans</i> 10261	Methionine	246	648
<i>C. albicans</i> 10259	—	790	1680
<i>C. albicans</i> 10259	Methionine	966	2960

The enzyme assay has been described previously.⁽⁴⁾

TABLE 10. ENZYMIC DECOMPOSITION OF ADENOSYLMETHIONINE BY EXTRACTS OF *C. albicans*

Enzyme source	Adenosylmethionine decomposed/ mg protein/hr, μ mole	
	Supplement in medium	
	None	Methionine
<i>C. albicans</i> 10231	52	40
<i>C. albicans</i> 10261	68	65
<i>C. albicans</i> 10259	157	173

The enzyme assay was the same as described previously⁽⁴⁾ except that the radioactive product is methylthioribose.

and 10261) and the mycelial culture (ATCC 10259) of *C. albicans* contain the enzyme adenosylmethionine (or methylmethionine):homocysteine methyltransferase. The cell-free extracts of the mycelial phase mutant (10259) form more methionine than do the cell-free extracts of the yeast phase cultures. Methylmethionine is much more efficient as a methyl donor than is adenosylmethionine. This may be due to the fact that both the yeast and the mycelial phases of *C. albicans* contain enzymes which decompose adenosylmethionine (Table 10). The adenosylmethionine decomposition enzyme may be in competition with the homocysteine methyltransferase and thus decrease the amount of methionine formed via the methyltransferase reaction. These results show that both the yeast and mycelial phases of *C. albicans* decompose adenosylmethionine in a manner similar to the enzymic decomposition which occurs with extracts of *A. aerogenes*.⁽⁴⁾

Evidence that this scheme occurs with cell-free extracts of *C. albicans* was obtained by using adenosyl-

TABLE 11. END-PRODUCT INHIBITION OF HOMOCYSTEINE METHYLTRANSFERASE OF *C. albicans*

Enzyme source	Methionine in reaction mixture, μ mole	Methionine/mg protein/hr, μ mole
<i>C. albicans</i>	0	240
	0.25	185
	0.5	155
	1.0	100
	2.0	67
	3.0	49
	4.0	37
	5.0	25

The enzyme assay has been described previously.⁽⁶⁾

methionine- $^{14}\text{CH}_3$ and adenosylmethionine-2- ^{14}C . When the $^{14}\text{CH}_3$ compound was used as the substrate with extracts from *C. albicans*, the principal radioactive product was methylthioribose with traces of methylthioadenosine. When adenosylmethionine-2- ^{14}C was used with extracts of *C. albicans*, homoserine was the only labeled compound observed with a chromatogram scanner.

The presence of the end product of a metabolic pathway in a growth medium often results in a repression of the enzymes required for the biosynthesis of that product. There was, however, an increase in the methyltransferase activity of cell-free extracts of *C. albicans* prepared from cells grown in the presence of 400 μ moles of methionine/ml (Table 9). Because of this, it was decided to explore the influence of methionine on homocysteine methyltransferase, *in vitro*. It may be seen in Table 11 that the addition of methionine to the reaction mixtures resulted in a marked inhibition of enzyme activity. The inhibition of methyltransferase activity by methionine was observed with cell-free extracts prepared from both yeast and mycelial phases of *C. albicans* grown either in the presence or absence of methionine. The presence of methionine in the growth medium did not appear to have any influence on the activity of the adenosylmethionine decomposition enzyme (Table 10).

It has been demonstrated that cystathionine substitutes for homocysteine as a methyl acceptor in the homocysteine methyltransferase reaction of *E. coli* and *A. aerogenes*.⁽⁵⁾ With the cell-free extracts prepared from the three strains of *C. albicans*, however, cystathionine did not substitute for homocysteine.

CONCLUSIONS

The production of methionine from homocysteine is catalyzed by cell-free extracts of *C. albicans* (yeast and mycelial forms) via a homocysteine methyltransferase reaction. It was also demonstrated that growth in the presence of methionine resulted in increased homocysteine methyltransferase activity. The apparent failure of methionine to repress homocysteine methyltransferase activity may be explained by the capacity of *C. albicans* to synthesize and store adenosylmethionine in its vacuoles. In addition to showing more methyltransferase activity than the yeast phase cultures, the mycelial mutant (ATCC 10259) also stores more adenosylmethionine in its vacuoles. Adenosylmethionine accumulation in the vacuoles is evident, even when no methionine is added to the medium, although larger quantities (75 μ moles/g dry weight) of adenosylmethionine are stored by the mycelial mutant when the growth medium is supplemented with methionine.⁽⁶⁾ Thus, the capacity to synthesize and store adenosylmethionine may remove any excess methionine within the yeast cells.

Methionine added to reaction mixtures results in inhibition of homocysteine methyltransferase. This inhibition of the enzyme, *in vitro*, suggests that little or no free methionine is available in the cytoplasm of the cell, even when methionine is added to the growth medium. The failure of cell-free extracts of the yeast and mycelial forms of *C. albicans* to use cystathionine, in place of homocysteine, as a methyl acceptor indicates that cystathionine is not an important precursor of homocysteine in *C. albicans*.

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A DOMINANT MUTATION FOR ETHIONINE RESISTANCE IN *SACCHAROMYCES CEREVISIAE**

Kemet D. Spence, Leo W. Parks,† and Stanley K. Shapiro

An ethionine-resistant mutant of *Saccharomyces cerevisiae* has been investigated whose mutation (*etr*²) confers resistance to the heterozygous diploid also containing the sensitive allele, *et*^s. The mutation is apparently specific for reversal of ethionine inhibition. The principal difference between the sensitive *et*^s strain and the mutant was the latter's inability to concentrate large intracellular quantities of adenosylethionine. Reduced incorporation of ethyl groups or ethionine in other cellular fractions of the mutant was also detected.

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The data show that the mutant has not lost the ability to form adenosylethionine. It is suggested that the mutant has an increased ability to hydrolyze this sulfonium compound after it has been synthesized. It is possible that some of the ethionine is detoxified before it can participate in either protein or adenosylethionine synthesis. No mutant alteration in accumulation of ethionine from the medium was detected. In the presence of ethionine, the parental strain accumulated 25 times more adenosylethionine than did the mutant. However, with methionine, only two times more adenosylmethionine was accumulated by the parental strain, as compared to the mutant.

SOME NOVEL MUTANTS OF *SACCHAROMYCES CEREVISIAE* INHIBITED BY ADENOSYLMETHIONINE AND ADENOSYLHOMOCYSTEINE*

Kemet D. Spence and Stanley K. Shapiro

These investigations have established the existence of a novel type of nonnutritional mutant (*ai*), which is inhibited in the presence of two naturally occurring cellular compounds. The inhibition is complete at an extracellular concentration at least as low as 0.05 μ mole/ml of either adenosylhomocysteine or adenosylmethionine. It is suggested that adenosylhomocysteine is the true inhibitor. The *ai* mutants are phenotypically indistinguishable from the wild type in the absence of inhibitors. The results have shown that if any direct effect on the methionine biosynthetic pathway exists, it is a secondary rather than the primary effect of the inhibitors. The *ai* mutation does not involve the loss of the adenosylmethionine (or methylmethionine):homocysteine methyltransferase. In addition, the *ai* mutants accumulate, maintain and utilize adenosylmethionine and methionine in a manner simi-

lar to that of the parental strain. No genetic relationship could be detected between the *ai*₁ mutation and several different markers affecting methionine biosynthesis. The *ai*₁ mutation was also shown to be genetically recessive. Methionine partially reverses the inhibition caused by adenosylmethionine or adenosylhomocysteine. Neither methylmethionine nor homocysteine reversed the inhibition which showed that the homocysteine methyltransferase cannot catalyze the synthesis of sufficient methionine under these conditions to simulate the effects of extracellularly supplied methionine. If adenine is present, methionine does not cause reversal of inhibition due to adenosylmethionine or adenosylhomocysteine. From the data presented, it is clear that the *ai* mutation involves some metabolic control mechanism, although the alteration does not appear to be associated primarily with the biosynthesis of methionine.

* Abstract of paper submitted for publication.

EFFECT OF WHOLE-BODY X IRRADIATION ON LIVER β -GLUCURONIDASE ACTIVITY

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PURPOSE AND METHODS

Hartiala et al.⁽¹⁾ indicated that 12 hr, and again 5–14 days, after 400–1200 R of whole-body X irradiation

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to rats, the β -glucuronidase activity of the liver showed an increase. Wishing to pursue the mechanism of these findings, we first attempted to repeat this work in mice. Male mice, approximately three months of age, were irradiated with 400 or 1200 R, with some

controls being left unirradiated. Six, 24, or 48 hr, or 5 days after the irradiation, groups of 4 mice were sacrificed, and the individual livers were assayed for β -glucuronidase activity by the method of Gianetto and deDuve.⁽²⁾ All mice were fasted for 48 hr before assay to minimize the glycogen turbidity that sometimes interfered with spectroscopy of liver preparations. In another experiment, groups of 10 mice were assayed 3, 6, 9, or 12 hr after 400 R of whole-body X irradiation.

PROGRESS REPORT

No consistent, significant effects of whole-body X irradiation were observed on the activity of mouse

liver β -glucuronidase, and the question is being pursued no further.

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STUDIES ON RAT LIVER RIBONUCLEASES. I. INTRACELLULAR LOCALIZATION OF THE ALKALINE RIBONUCLEASES*

Yueh-Erh Rahman

Studies on the intracellular localization of rat liver alkaline ribonucleases have been completed. Results obtained from various fractionation methods, such as differential centrifugation, zonal centrifugation, and

* Abstract of a paper published in *Biochim. Biophys. Acta* **146**, 477 (1967).

sucrose density-gradient centrifugation showed that both alkaline ribonucleases in rat liver were diffusely distributed in all subcellular fractions; RNAse II (pH 8.0) had higher activity in the supernatant than RNAse III (pH 9.5). This diffuse distribution pattern is not due to adsorption phenomena.

STUDIES ON RAT LIVER RIBONUCLEASES. II. PARTIAL PURIFICATION OF THE ALKALINE RIBONUCLEASES FROM MITOCHONDRIA

Yueh-Erh Rahman

PURPOSE AND METHODS

Results reported in section I of this series indicated that both alkaline ribonucleases found in rat liver were diffusely distributed in all subcellular fractions obtained by various centrifugation methods. In order to study the enzymatic function of these ribonucleases directly, these enzymes had to be separated from various subcellular fractions. Since the mitochondrial fractions were found to contain between 30 and 40% of both alkaline ribonucleases,⁽¹⁾ attempts were made to isolate these enzymes from the mitochondria by the following procedure:

1. Subfractionation of mitochondria: Mitochondrial fractions were subfractionated to obtain the inner and outer mitochondrial membranes by either the method of Sottocasa et al.⁽²⁾ or that of Schnaitman et al.⁽³⁾
2. Solubilization studies of ribonucleases from mitochondrial fractions: Portions of the same mitochon-

drial preparation were resuspended in the following various media: sucrose, water, deoxycholate (0.1%), Triton X-100 (0.1%), or digitonin (0.1%) or were sonicated for 8 min, and were then centrifuged in the Spinco 50 rotor of the Model L-2 centrifuge at 226,000 *g* for one hour. Ribonuclease assays were performed on these samples before centrifugation, as well as on the supernatant and the precipitate obtained after centrifugation.

3. Ribonucleases were purified from mitochondrial fractions by the use of deoxycholate and trypsin digest.

PROGRESS REPORT

By the method of Sottocasa et al. an average of 80% of both alkaline ribonucleases were obtained from the inner membrane fractions of the mitochondria, while the Schnaitman's method yielded 60% for both enzymes.

Results obtained with solubilization studies are shown in Table 12.

The purification of mitochondrial alkaline ribonucleases using deoxycholate and trypsin digest was not successful. This failure was due to the ineffectiveness of the trypsin in solubilizing the ribonucleases from the mitochondrial membranes, since near 60% of both alkaline ribonucleases remained in the P_2 fraction (see Figure 64).

CONCLUSIONS

1. In rat liver mitochondria, both alkaline ribonucleases are found to be localized mainly in the inner membranes.

2. The two alkaline ribonucleases are tightly bound to the inner membrane, the use of various detergents, trypsin, and sonication were ineffective for their complete solubilization.

3. The acid ribonuclease (RNAase I) has a different intracellular localization, it is readily solubilized by the treatments indicated in Table 12.

Both alkaline ribonucleases are tightly bound to the inner membranes of rat liver mitochondria, and further studies will be needed before their possible functions, as related to the nucleic acid metabolism within the mitochondria, can be understood. New methods should be tried for obtaining complete solubilization of these ribonucleases, since this is a primary step in the purification of these enzymes.

TABLE 12. ENZYME ACTIVITY FOUND IN SUPERNATANT

	RNAase I	RNAase II	RNAase III
	% of Total activity before centrifugation		
Sucrose (0.25 M)	4	10	11
Water	66	17	11
Deoxycholate (0.1%)	80	22	9.5
Triton X-100 (0.1%)	54	17.5	16
Digitonin (0.1%)	2 (?)	2.5	3.5
Sonication (8 min)	65	15	10

STUDIES ON RAT LIVER RIBONUCLEASES. III. STUDIES ON BIOLOGICAL FUNCTIONS OF RAT LIVER RIBONUCLEASE

Yueh-Erh Rahman

PURPOSE AND METHODS

Acid ribonuclease was reported by de Duve et al.⁽¹⁾ as one of the lysosomal enzymes; it was found, along with other acid hydrolases, to be involved in most

FIG. 64.—Summary of partial purification of alkaline ribonucleases from mitochondria.

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physiological involution and pathological degenerations of mammalian tissues.⁽²⁾ RNAase II was reported, on the other hand, to be involved in the breakdown of messenger-RNA in rat liver;^(3, 4) the

implication of a diffuse distribution pattern of this ribonuclease⁽⁵⁾ requires investigation. The possible biological functions of RNAase III are entirely unknown. In view of the importance of these enzymes in relation to cellular nucleic acid metabolism, on one hand, and the new findings of their intracellular localizations,^(5, 6) on the other, studies of all three ribonucleases are in progress on SD/Anl [Anl 6] rats under various treatments, including starvation, hepatectomy, adrenalectomy, and administration of carcinogens (i.e., thioacetamide).

PROGRESS REPORT

TABLE 13. SUMMARY OF RIBONUCLEASE ACTIVITIES IN LIVER OF SD/Anl [Anl 66] RATS UNDER VARIOUS TREATMENTS^(a)

	RNAase I	RNAase II	RNAase III
Fasting (24 hr)	0	0	0
Starving (5 days)	0	0	0
Actinomycin <i>in vitro</i> (0.001 mg/ml)	0	0	0
Actinomycin <i>in vivo</i> (1 mg/kg rat/24 hr)	-20	0	-19
Adrenalectomy	+20-30	0	+20
Thioacetamide <i>in vitro</i> (0.02 mg/ml)	0	0	0
Thioacetamide <i>in vivo</i> (S.C. inj. 50 mg/kg rat for 4 days)	0	0	0
Thioacetamide (0.4% mixed in food, 1 day to 15 days)	0	0	0

^(a) Expressed as % change related to untreated control rats; averages of results obtained from 3 to 5 separate experiments.

CONCLUSIONS

The enzyme activity of all three ribonucleases is unchanged under most treatments studied so far. RNAase I and the RNAase III, however, seem to change with the same pattern and in the same proportion; more work is needed before this relationship can be clarified. This relationship might be important to understand the biological functions of the RNAase III.

Other treatments, such as hepatectomy, X irradiation, and carcinogens other than thioacetamide, as well as hepatotoxin administrations, will also be examined.

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Isotope Studies

DETECTION AND ANALYSIS OF THE UNRESOLVED MONOTRITIUM-LABELED FORMS OF LEUCINE-4,5-³H ON ION EXCHANGE COLUMNS

Vaclav Cejka, Sylvanus A. Tyler, Merlin H. Dipert, and Peter D. Klein

PURPOSE AND METHODS

The problem of establishing radiochemical identity and purity by indirect means, in which primary parameters such as ultraviolet or infrared spectra, melting point, optical rotation and other indices of character cannot be used, is a recurrent and critical one.^(1, 2) In particular, it is essential to know, not only the chemical purity of the material used, but also the distribution of radioactivity within the molecule. During chromatographic studies of labeled amino acids, the mobility of leucine-4,5-³H was compared with that of leucine-1-¹⁴C. In addition to the difference in mobil-

ity between these two species, a larger value for the dispersion of the tritium peak was observed. Since individual species of the same molecule are subjected to comparable dispersion forces as they migrate down the column, the broader tritium peak indicates the presence of at least one other component. This report is concerned with evidence, (1) for the presence of two forms of tritiated leucine, (2) for the likelihood that these forms are present in equal quantities, and (3) for the quantitative estimation of the difference in mobility between the two forms. This evidence was acquired by the use of computer programs that permit

the direct digital synthesis of single and multicomponent peaks as well as techniques for the analysis of Gaussian distributions.

PROGRESS REPORT

The procedure by which L-leucine-4,5-³H is produced commercially involves the catalytic hydrogenation of L-dehydroleucine, which in turn has been isolated from the racemic DL-mixture prepared by condensation of methallyl bromide with the acetamido head group. Thus, the synthetic origin of the product does not include the possibility that isoleucine isomers are present. The optical activity of the immediate precursor provides proof that only the L-form is being hydrogenated and unreacted dehydroleucine can be removed without difficulty, leaving a single *chemical species*. On the other hand, even carrier-free tritium gas undergoes dilution by hydrogen adsorbed on the catalyst, and only in unusual circumstances can one expect to obtain two tritium atoms in each molecule of hydrogenated product. A far greater likelihood is that radioactivity will be randomly attached to either the 4 or 5 position.

To our knowledge, no studies have been carried out that predict the behavior of an enveloping peak as a

FIG. 65.—Relationship between band broadening and displacement between two component peaks.

TABLE 14. DOUBLET SEPARATION FACTOR FOR LEUCINE-4,5-³H FROM ³H/¹⁴C AND ³H/MASS MEASUREMENTS

Reference mobility	Reference dispersion	Tritium dispersion	Δσ, %	ΔM', %
¹⁴ C				
142.661	1.387	1.625	17.16	1.172
143.157	1.370	1.597	16.57	1.133
143.075	1.392	1.610	15.66	1.119
143.313	1.436	1.717	19.57	1.294
148.730	1.433	1.762	22.96	1.353
147.614	1.472	1.719	16.78	1.189
147.662	1.484	1.735	16.91	1.204
148.160	1.481	1.770	19.51	1.134
				1.200 ± .028
Mass				
142.669	1.397	1.605	14.89	1.057
148.243	1.452	1.660	14.32	1.075
147.046	1.267	1.588	24.78	1.258
147.613	1.372	1.588	15.74	1.072
147.656	1.385	1.677	21.08	1.259
				1.144 ± .047

TABLE 15. FITTING OF LEUCINE-4,5-³H AS A DOUBLET PEAK

	Synthetic composite input	Synthetic composite output	Observed experimental
<i>Mobility</i>			
Mass	147.046	147.046	147.046
Tritium	145.769	146.718	146.723
	147.648		
<i>Dispersion</i>			
Mass	1.267	1.267	1.267
Tritium	1.267	1.588	1.581
<i>Displacement—ΔM, %</i>			
Probit		−0.223	−0.219
Isotope ratio-1		−0.189	−0.024
Isotope ratio-2		−0.303	−0.315

function of the two component peaks comprising it, when the component peaks are of the same size and dispersion. Accordingly, a series of Gaussian peaks of identical area and dispersion were constructed by the computer with a progressive increase in the separation between the midpoints of the two component peaks. The combined peak was analyzed by the previously developed method of probit analysis to obtain the joint midpoint and its dispersion. Such series were constructed for a variety of original dispersions, and the results from all such series could be subsumed under the single relationship shown in Figure 65. According to this relationship, the growth in the dispersion of the joint peak is directly related to the distance separating the component peaks divided by the dispersion of the component peaks. This means that if the disper-

FIG. 66.—Tritium specific activity across chromatographic peak of leucine-4,5- ^3H . Open circles, experimental points; solid points, specific activity values computed from input parameters in Table 15.

sions of the component peaks are known, or can be estimated, the separation between the two unresolved peaks can be calculated from the dispersion of the combined peak. Exactly such a value is provided by the dispersion of the ^{14}C -labeled leucine, or from the dispersion of the unlabeled leucine peak as determined from probit analyses of the ninhydrin measurements. Table 14 lists the reference mobility and dispersion using either the ^{14}C or mass peak, the corresponding tritium dispersion, and the displacement required between two components to give rise to the increased dispersion. Excellent agreement is obtained between these two

series as evidenced by the small uncertainty of the estimate. When these values are used to compose the synthetic analogue of the experimental peak, as shown in Table 15, the resultant dispersion of the mixed peak and its displacement from the reference peak agree remarkably well with experimental observations. Finally, the behavior of the tritium specific activity predicted by the computations corresponds closely to those values measured directly. This is seen in Figure 66. We conclude from this that the proportion of material in the two forms, leucine-4- ^3H and leucine-5- ^3H , must be closely similar.

CONCLUSIONS

Commercial leucine-4,5- ^3H appears to consist of two equal populations of leucine-4- ^3H and leucine-5- ^3H with mobilities differing on ion exchange columns by 1.2%. Recognition and analysis of this mixture has opened the possibility of resolving other distributions whose components are known to follow a Gaussian pattern but in which there is an extensive overlap between two such distributions. Higher-order multiplet peaks are also under investigation using the experience gained in this study.

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A COMBUSTION METHOD TO ASSAY CARBON-14, TRITIUM, AND DOUBLE-LABELLED BIOLOGICAL MATERIALS

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PURPOSE AND METHODS

Biological and medical investigations, in practice, call for counting systems that have the greatest versatility. One of the more important requirements is that these systems must analyze large numbers of samples with a minimum of preparation. With the continued and increasing use of the low-energy beta emitters, e.g., carbon-14, sulfur-35, and tritium, a large sample load can be handled effectively only by liquid scintillation counting. In most cases, sample preparation time is comparatively short, and the automatic sample changers, holding up to 200 samples, require a minimum of attention once the initial electronic conditions

are set up. The newer units, which feature automatic counting of each sample with an external standard for efficiency determination, are particularly advantageous under such conditions. The increasing availability of computational facilities, resulting in specific computer applications for radioisotope experiments in which data are obtained by liquid scintillation counting, should be included in this advantage.

On the other hand, the chief limitation involved with liquid scintillation counting is sample dissolution and quenching. The term "quenching" covers several similar effects, all of which result in a decreased counting efficiency for the system. In measuring various bio-

FIG. 67.—Diagram of direct in-vial isotope collection apparatus for double-labeled samples. A, Schematic diagram of the combustion apparatus and collection assembly for H_2O ; B, collection assembly for CO_2 or SO_2 .

logical materials one is faced with colored compounds, or those compounds that can absorb radiation energy more efficiently than the liquid scintillation phosphor. This causes a decrease in counting efficiency of any compound being counted. The net effect is that not all samples show the same counting efficiency in a given phosphor, and the efficiency must be determined for each sample material or class of compound.

If the color quenching is not excessive, it can be corrected numerically by one of three ways: (1) by adding an internal standard, usually labeled toluene, (2) by observing the ratio of counts in two different energy channels because quenching shifts the energy spectrum to lower levels, and (3) by counting each sample with a built-in external standard source after each initial count. If the color quenching is excessive, however, and/or the sample material is varied and possibly difficult to dissolve in the phosphor, there is no alternative but complete oxidation of the sample.⁽¹⁾

Dry catalytic combustion at high temperatures is probably the most accurate method for combusting these materials, and it has been used for carbon-14, sulfur-35, and tritium. A modification of the Schoniger oxygen flask technique⁽²⁾ provides for convenient combustion of dried biological materials or organic compounds with the quantitative collection of the resultant carbon dioxide, sulfur dioxide, and water for subsequent liquid scintillation measurement of carbon-14, sulfur-35, and tritium. Our experimental efforts have been directed toward developing a technique that combines the simplicity and facilitation of the oxygen flask method with the increased efficiency and reproducibility of standard vacuum line techniques, in-

TABLE 16. TRITIUM RECOVERY BY COMBUSTION

Tissue weight, ^(a) mg	Intestine		Liver		Blood	
	Rec., %	QF	Rec., %	QF	Rec., %	QF
14	97.8	0.975	98.5	0.978	99.2	0.977
25	97.8	0.982	99.1	0.974	96.6	0.984
50	99.0	0.976	97.5	0.971	98.1	0.971
100	98.8	0.957	97.8	0.959	96.9	0.966
	Av. 98.4		Av. 98.2		Av. 97.7	

^(a) Std. control (30 + 660 cpm) 100%, quenching factor (QF) 0.996. Std. combustion control recovery (Rec.) %, 98.1; QF 0.988.

cluding the convenience of a direct in-vial collection of the final products of combustion.

PROGRESS REPORT

The apparatus used in these investigations is shown in Figure 67. The apparatus, somewhat modified from that previously reported,⁽³⁾ consists of a 500-ml combustion flask housed in a heating jacket, a spiral cold trap (A) used for water collection and a dual trap (B) used for carbon dioxide collection. A standard scintillation vial is attached to each assembly via a specially constructed connector and copper-to-glass seal. In the case of double-labeled samples, such as carbon-14 and tritium, the cold traps (A) and (B) are connected in series and the vacuum pump is connected to trap (B).

Recovery values for tritium from different tissues, with sample weights up to 100 mg of dry tissue weight, are shown in Table 16. Recovery values average 98%

TABLE 17. SEPARATION EFFICIENCY FOR CARBON-14 AND TRITIUM BY COMBUSTION

Standard added, cpm		Liver, mg	% Recovered	
³ H	¹⁴ C		³ H	¹⁴ C
0	6.2×10^4	0	—	98.6
3.8×10^4	0	0	97.4	—
"	6.2×10^4	0	96.1	98.0
"	"	10	97.2	98.7
"	"	20	97.4	98.2
"	"	30	97.8	99.1
"	"	40	97.5	99.2
"	"	50	97.6	98.8

and are reproducible and independent of both the amount and the nature of the tissue analyzed. Though the quenching factor increases slightly with increasing tissue weight in all tissues, it is constant and reproducible at any tissue weight used for analysis. This would indicate that where accuracy of a few percent can be tolerated, or where only relative counting rates are required, consideration of quenching could be omitted.⁽⁴⁾

The separation efficiency for carbon-14 and tritium, and recovery values for carbon-14, are shown in Table 17. The recovery values for tritium and carbon-14 are seen again to be approximately 98%. The separation efficiency is quite satisfactory, giving recovery values similar to those observed when only the single isotope is measured.

CONCLUSIONS

This method of analysis allows for the collection of the combustion products, H₂O and CO₂ directly into standard scintillation vials, giving quantitative recovery of tritium and carbon-14. In double-labeled

compounds, a separation of the active combustion products is also quantitatively achieved.

In addition to overcoming the problems of sample solubility and quenching limitations of liquid scintillation counting, this technique has certain other advantages. For most samples, assays can be carried out without recourse to an internal standard to give results of moderate accuracy, to within 5%. This is possible because the makeup of the final scintillation solution is the same for each sample, regardless of the chemical nature of the original sample.

The main advantage of this combustion technique for counting double-labeled samples is that only a single channel is necessary for counting, and instrument settings and calibration procedures are not critical, and can be optimized for each isotope to be counted. Since the isotopes in question can be completely separated, the range of ratios of one isotope to the other that can be determined by this technique is far greater than is possible with methods based on differences in energy spectra. Furthermore, only a simple mathematical analysis of the data is necessary, since quenching corrections are eliminated.

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THE PREPARATION OF TRITIUM-LABELED BILE ACIDS BY EXCHANGE-LABEL CHROMATOGRAPHY ON TRITIATED ALUMINA

Alan F. Hofmann, Patricia A. Szczepanik, and Peter D. Klein

PURPOSE AND METHODS

Earlier, we reported that tritium can be incorporated into molecules of biological interest by relatively simple expedients. These tritiated compounds possess considerable utility and have opened new areas of study. As part of a collaborative program with investigators in other fields to prepare labeled compounds and devise methods for their production, we synthesized tritium-labeled bile acids by techniques previously

used for ketosteroids⁽¹⁻⁴⁾ and report here the successful synthesis of a variety of labeled bile acid metabolites.

PROGRESS REPORT

Cholic acid, (3 α ,7 α ,12 α -trihydroxycholic acid) whose structure is shown in Figure 68, is one of the four major bile acids excreted by mammals; the others are chenodeoxycholic acid (3 α ,12 α -dihydroxy cholic

FIG. 68.—Cholic acid

acid) deoxycholic ($3\alpha,7\alpha$ -dihydroxy cholanic acid) and lithocholic acid (3α -hydroxycholanic acid). Commercial radioactively labeled bile acids are usually derived from the parent acid by decarboxylation and reaction of the halide derivative with a Grignard product containing radioactive CO_2 . The product is expensive and its activity is limited by the available specific activity of $^{14}\text{CO}_2$. An alternative route of radiosotope labeling, using tritium, was sought, and the following procedure was developed: 1) oxidation of the bile acid methyl ester to the 3-keto derivative; 2) conversion of the free hydroxyl groups to acetoxy groups by acetylation with acetic anhydride; 3) chromatography of the keto bile acid derivative on alumina deactivated with tritiated water, resulting in enolic exchange of hydrogens on either side of the ketone with tritium on the alumina surface; 4) borohydride reduction of the ketone to a mixture of α and β epimeric hydroxyl groups; 5) removal of the acetoxy groups, and 6) separation of the epimers by thin layer chromatography.

This labeling procedure has been successfully applied to each of the bile acids listed above, and products having specific activities of 2 to 4 mCi/mM have been obtained without difficulty. Analysis of the radiochemical purity has shown that the contamination by the isomer in the final product is usually under 3%, indicating adequate resolution of the epimers by the thin layer chromatogram. However, extensive losses in the overall preparative yield were sustained during the steps required to remove the blocking acetoxy groups in the di- and trihydroxy bile acids, and alternative tactics were investigated. Blockage of the hydroxyl groups was originally considered necessary to enable elution of the bile acid derivative by solvents of low polarity; the free hydroxyl groups greatly increase the absorption of the molecule on the alumina surface, and

polar solvents must be used for elution. These solvents are usually hydroxylic in nature (e.g., methanol and chloroform), and their use must be avoided since they will exchange protons with the surface and strip it of all radioactivity. Additionally, any polar solvent, whether or not it is hydroxylic in nature, may displace the water adsorbed on the alumina surface and thus deactivate the column.

It was possible to elute the deoxycholic and chenodeoxycholic acids with chloroform-benzene solutions and to elute cholic acid with ethyl acetate when the 3-keto derivatives were applied as their methyl esters, thereby eliminating steps 2 and 5 from the procedure described. In essence, it is possible to chromatograph the keto derivatives, reduce them to alcohols and remove the 10–15% of the β isomer by thin layer chromatography in less than a day. Overall yields of the bile acids are 60–70% of the starting keto compound. These products have been used in studies of bile acid absorption in the human intestine; their availability makes it possible to study free acid-conjugate relationships using ^{14}C - ^3H combinations; alternatively, the metabolism of cholic and chenodeoxycholic acid can be followed in the same patient by dual-label techniques.

CONCLUSIONS

2,4- ^3H -labeled lithocholic, chenodeoxycholic, deoxycholic and cholic acids were prepared by exchange-label chromatography of their 3-keto derivatives on tritiated alumina and by their reduction to the corresponding alcohols. These tritium-labeled bile acids are convenient to prepare, readily obtained in a radiochemically pure form, possess adequate activity for metabolic studies and require only tritiated water as the source of radioactivity.

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GAS CHROMATOGRAPHY AS A METHOD OF INTRODUCING AMINO ACID SAMPLES INTO A MASS SPECTROMETER

Peter D. Klein, Frank Mannino and Patricia A. Szczepanik

PURPOSE AND METHODS

The analysis of isotope fractionation data on amino acids has shown that there are a variety of positional effects on the chromatographic mobility on ion exchange columns.⁽¹⁾ Investigation of these effects and their correlation with intramolecular forces operating in the ion exchange process is limited by the availability of tritium- and radiocarbon-labeled compounds from commercial sources. Furthermore, radiochemical hazard and stability problems limit the range of isotope concentrations that can be studied when beta-emitting isotopes are used to trace the migration of the amino acid. The use of stable isotopes, on the other hand, permits study of concentration dependent phenomena as well as of the contributions of nitrogen and oxygen isotopes. Stable isotopes have held little interest in recent years for most analytical users because of the necessity of combusting each sample, or of introducing it as a solid in the electron beam of the mass spectrometer. Recently, however, the successful connection of a gas-liquid chromatograph to the inlet of a mass spectrometer has made possible the direct introduction of virtually any sample into a mass spectrometer, provided only that it has sufficient volatility to be analyzed or separated on a gas chromatograph. The block diagram shown in Figure 69 illustrates the components of the system being assembled. In operation, the sam-

ple (or mixture) is introduced into the gas chromatograph and is fractionated by the chromatograph into its constituent compounds. The compounds (as volatile derivatives) are carried into the mass spectrometer after passing through a molecular separator which strips out 99.9% of the carrier gas. In the spectrometer ion source, the molecules are ionized and accelerated through a magnetic field which distributes them according to mass number. The mass number is scanned by the instrument, and ion intensities at each mass number are recorded on either magnetic tape or are converted to digital values and stored according to mass number in a multichannel analyzer. At the same time, the total ion current (proportionate to sample size) is monitored to indicate the emergence of each sample component. The sample peaks so recorded are translated into digital values by the digital peak integrator. These values and those accumulating in the multichannel analyzer are routed through a multiplexing terminal to the key punch. The multiplex permits sequential or programmed use of the same key punch by two or more output devices. Processing of either the magnetic tape record or the information on punch cards is carried out using the GE 225 facilities in the building. This report is concerned with the investigation of gas chromatography of amino acids as a means to introduce them into a mass spectrometer and thereby determine their stable isotope content.

FIG. 69.—Components of integrated gas-chromatograph mass spectrometer system. Solid lines, existing components; dashed lines, equipment under construction; dotted lines, components to be installed.

ROGESS REPORT

Assembly and debugging of this system is being carried out from the front end, beginning with the preparation of volatile amino acid derivatives, their analysis by gas chromatography and integration of the chromatogram record. The *N*-trifluoroacetyl *n*-butyl ester was selected as the best derivative to render the amino acids volatile. Using the procedure of Gehrke and Stalling,⁽²⁾ the dry amino acid is esterified in a solution of *n*-butanol-hydrogen chloride to form the *n*-butyl ester hydrochloride. This is then acylated with trifluoroacetic anhydride to form the double derivative in which both the amino and carboxyl groups are blocked. Most amino acids of interest can be eluted from a gas chromatographic column (whose stationary phase is composed of ethylene glycol succinate-methyl silicone polymer and ethylene glycol succinate) in a temperature range of 95–150° C within 3 to 5 min.

In practice, the following problems have had to be overcome to render the procedure useable. First, it could be anticipated that the procedure would involve 20 to 40 samples per run, and a means of handling these numbers of samples through the reaction sequence had to be devised. Small screw-cap vials with Teflon liners were employed as the reaction vessels; drying, solvent removal, and reactions at elevated temperatures were carried out in a specially constructed heater block fitting the inside of a desiccator. Second, quantitation of submicrogram levels of amino acids through a sequence of reactions and several transfer procedures required the development of an internal standard technique. Satisfactory results were obtained by using a second amino acid of known con-

centration added to each column fraction after aliquoting into the reaction vials. Reproducibility and recoveries were entirely adequate down to levels of 20 nanograms of injected sample. Third, the usual citrate buffers used to elute the columns proved to contain contaminants at the microgram level which interfered with the detection of the amino acid peaks. Substitution of ammonium formate buffers not only eliminated the contaminants but permitted removal of the buffer salts by sublimation of the ammonium formate at reduced pressures. Fourth, the original determinations of peak area carried out by planimeter proved to be the step with the greatest time consumption. Installation of a digital peak integrator, recently completed, has increased both the speed and accuracy with which the analyses can be carried out. The values so obtained appear to have greater reproducibility than conventional colorimetric reactions using ninhydrin, while requiring one fourth as much sample. Computer programs to convert relative peak areas into absolute quantities of amino acid and the introduction of these values into existing programs for computation of isotope fractionation are being developed by David R. Frye. Introduction of the molecular separator and mass spectrometer system will begin the next stage of system assembly.

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THE INFLUENCE OF WATER STRUCTURE ON ISOTOPE FRACTIONATION IN ION EXCHANGE COLUMNS

Peter D. Klein and Patricia A. Szczepanik

PURPOSE AND METHODS

The measurement of isotope fractionation, during ion exchange chromatography of labeled amino acids, has been a major interest in our laboratory. Measurements of the fractionation of ³H- from ¹⁴C-labeled amino acids and their displacement from unlabeled varieties of the same molecule have been carried out for 37 examples, and the results have been reported in these reports and elsewhere.^(1, 2) During these studies, it was noticed that the displacement measured between a given pair of ³H and ¹⁴C forms of an amino acid was

perceptibly influenced by the presence of unlabeled amino acid, being increased in magnitude over that observed in the absence of added amino acid material. The exceedingly small quantities (2.5 μM) of material involved in these effects suggested that local ordering of the solvent in the vicinity of the migrating species was affected by the total quantity of amino acid in the sample. An extensive investigation of the relationship between concentration and displacement observed is not practical for two limiting reasons: (1) the specific activity of commercially available labeled amino acids at

FIG. 70.—Probit analyses of tritium- and radiocarbon-labeled tyrosine species chromatographed in the presence and absence of added unlabeled tyrosine. Open circles, tritium; solid circles, radiocarbon.

the low end and (2) the overloading of the column at the upper end of the range. An alternative tactic is to use agents known to disrupt water structure and to measure the displacement between isotopic species with and without added mass in buffers that contain such agents.

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An illustration of the effect of added unlabeled amino acid on the separation between a ^3H and ^{14}C pair of radioactive tyrosine compounds can be seen in Figure 70. The cumulative percent of material eluted plotted as the probit transform against the elution volume for tyrosine- $3', 5'-^3\text{H}$ and tyrosine- $1-^{14}\text{C}$ in the presence and absence of $2.5 \mu\text{M}$ of unlabeled tyrosine. The radioactive forms together contributed $0.192 \mu\text{M}$. The difference in mobility (separation) between the tritium and radiocarbon is indicated by the horizontal distance separating the midpoints of each line. This distance is increased almost 50% by the addition of mass. Similar, but less extensive, changes were found with every other pair tested, and in no instance was there a decrease in the separation when unlabeled material was present. A least squares analysis of the relationship between Y , the separation with added mass,

and X , the separation in its absence, gave a linear equation whose intercept A did not differ significantly from zero, and whose slope B yielded the value 1.211 ± 0.062 . The latter is significantly greater than the value of 1.00, which would be expected if the changes were randomly distributed in positive and negative fashion and indicates that the increase in separation is proportionate to the original magnitude of the separation.

Tertiary butanol was selected as the agent most suitable for disruption of local ordering in the buffer solutions. Its effect is maximal at a concentration of 4 molecules percent (or about 12 volumes percent), it is completely miscible with the buffers, and it does not interfere with ninhydrin determinations. Accordingly, buffers of the same ionic strength and pH were made up with aqueous solutions of tertiary butanol. To date, nine pairs of tritium- and radiocarbon-labeled amino acids have been studied in chromatograms with and without added unlabeled material. The retention volumes of the amino acids are reduced 15 to 30% by the tertiary butanol, with the aromatic amino acids being affected the most. In no instance was there a difference in the separation between ^3H and ^{14}C amino acids when unlabeled amino acid was added. Plotting the separations with and without mass against each other, as previously, yielded a linear relationship where slope was 0.982 ± 0.036 , indicating the absence of any amino acid concentration effect on the separation, even at large separation values. Alternatively and more important, the effect observed in aqueous systems is a strong indication that local solvent structure is present in the chromatographic buffers. This structure appears to restrict the free diffusion and separation of the two labeled species, and its effect is proportional to the degree of separation existing between the species.

Structuring of water is usually regarded as strongly temperature dependent, and at 50° , the temperature at which these columns were run, it is surprising to find any evidence of its presence remaining. On the other hand, the difference in mobility of ^3H - and ^{14}C -labeled tyrosine represents a difference in the free energy of interaction with the resin of only 4 cal/mole. Such small differences are easily affected by even a slight deviation from ideality in solution and may indeed serve to probe the extent of such deviation.

CONCLUSIONS

Measurements of isotopic fractionation during ion exchange chromatography of labeled amino acids indicate the presence of local structure and order in the aqueous buffers used for elution. Although this effect is

small, it can be measured with accuracy and it can be abolished by the incorporation of known structure-breaking compounds in these buffers. This interaction with the isotopic fractionation process suggests that the temperature dependence of water structure can be assessed by the chromatography of dual-labeled amino acids with and without carrier amino acid.

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TRANSPORT OF INGESTED LIPIDS FROM THE BLOOD TO ADIPOSE TISSUE, LIVER, AND MUSCLE STUDIED BY ELECTRON MICROSCOPIC AUTORADIOGRAPHY

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PURPOSE AND METHODS

This study was undertaken to both complement and extend earlier descriptive studies of the transport of lipids from the blood to the fat cells.⁽¹⁾ We are tracing tritium-labeled palmitic acid from the intestinal tract

of rats to the cells of their adipose tissue, liver, and muscle by electron microscopic autoradiography. We expect to determine not only the pathways by which this fatty acid is distributed in these tissues, but also the relative amounts taken up by cells of these different tissues in relation to time.

Starved male albino SD/Anl [Anl 66] rats, weighing about 200 g (weight loss about 30% of original body

FIG. 71.—Cross section of a capillary with erythrocytes. Connective tissue (collagen fibrils) separates the wall of the capillary from adjacent fat cells. At the top of the capillary in this figure is one of the long processes characteristic of depleted fat cells, with finger-like extensions into the connective tissue, while in the right upper and left lower corners of the photograph, larger fat droplets surrounded by a rim of cytoplasm are seen. The radioactive material, represented by the tortuous "grain," (arrow) is located in the capillary wall evidently on its way out into the connective tissue. FC, fat cell; CF, collagen fibrils; CW, capillary wall; CL, capillary lumen; E, erythrocyte. Specimen taken 24 hr after applying labeled palmitic acid. Exposure time 5 weeks. 26,800 $\times$.

FIG. 72.—This figure shows two fat cells; of the one at the right, only the part that contains the nucleus is seen; the other cell is cut through the middle of a large fat droplet. Notice the characteristic surface of the partly depleted fat cell. The grain (arrow) lies over the intercellular space; it seems to represent a radioactive element on its way to one of the fat cells. FC, fat cell; FD, fat droplet; CF, collagen fibrils. Sample taken 2 hr after application of the labeled fatty acid. Exposure 5 weeks. 26,800 $\times$.

weight), were given 1 mCi tritium-labeled palmitic acid in 2 ml of olive oil by stomach tube. At intervals of 2, 4, 24, 48, and 96 hr after treatment, the tissues were collected for both radiochemical (Kisielecki) and electron microscopic (Wassermann, Manelis) investigations. To prepare the tissues for electron microscopy they were fixed in 2½ or 3% glutaraldehyde for 24 hr at about 6°C, washed and then divided into parts of 1 mm (side length) for postfixation in 1% osmic acid (on ice) for 2 hr. They were then dehydrated, embedded in Epon, and sectioned with a Porter-Blum microtome. These sections were stained with uranyl acetate and covered with carbon film. In the dark room, liquid Ilford Nuclear Research Emulsion, Type L4, was applied to the grids by the wire loop technique. Exposure time (in the refrigerator) varied from 10 days to 8 weeks. After they were developed (Kodak Microdol), fixed, washed, and dried, sections were stained for a second time with lead acetate and studied

FIG. 73.—This figure shows the long branching processes of a depleted fat cell surrounded by connective tissue. Two grains (arrow) are seen over the cytoplasm, which means that the radioactive substance has entered the cytoplasm of the fat cell. FC, fat cell; CF, collagen fibrils. Specimen taken 4 hr after applying the active material. Exposure time two months. 22,500 ×.

FIG. 74.—A fat cell with a long process in the diagonal of the picture. The radioactive material (arrow) is settled in the cytoplasm, as in Figure 73. FD, fat droplet; C, cytoplasm; CF, collagen fibrils. Sample taken at 24 hr. Exposure time one month. 26,800 ×.

with an RCA-3F electron microscope. Tissues from rats that received only olive oil were treated in the same way as the radioactive tissues and served as controls.

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Radiochemical Analysis

Radioactivity was identified⁽²⁾ in all tissues at 2 hr; the maximum concentration was reached at 24 hr. At 96 hr, significant amounts of radioactivity were still detected in all tissues. The concentration of radioactivity in adipose tissue at 2 hr was 4×10^{-4} μ Ci/mg of dry tissue, while at 24 hr its concentration was 4 times that detected at 2 hr. The level of radioactivity in the liver was, up to 24 hr, significantly higher; in muscle, the values were similar to those observed in adipose tissue.

Electron Microscopy

Only the findings in adipose tissue are presented in this progress report. Since the tritium-labeled palmitic acid continues to move from the blood to the fat cells

FIG. 75.—This figure shows part of a fat cell enclosing a medium-sized fat droplet fusing with a smaller one. The grain (arrow) is at the edge of the fat droplet, which suggests that the radioactive material is taken into the stored fat. C, cytoplasm of the fat cell; FD, fat droplet; CF, collagen fibrils; cap, capillary. Sample taken 48 hr after applying the labeled fatty acid. Exposure time 10 days. 26,500 $\times$.

throughout our experiment (96 hr), the electron micrographs are not arranged according to the time after it was administered to rats, but are arranged according to its localization in the tissue. In this way, we are able to demonstrate how we trace the pathway of this fatty acid by radioautography.

In Figure 71, a grain is seen over the wall of a capillary that is close to a fat cell. Evidently, the lipid is caught passing through the capillary wall as it enters the connective tissue. Grains inside the capillary lumen also have been observed.

The grain in Figure 72 is located in the connective tissue between two fat cells; one is sectioned tangentially, showing only the part of the cell with the nucleus, while the other is cut through the middle of one large and one small fat droplet.

In Figures 73 and 74, the active material has entered the cytoplasm of the fat cells, while in Figures 75 and 76 it joins already established fat droplets. This illus-

FIG. 76.—This figure shows grains inside a larger fat droplet, demonstrating the incorporation of recently-taken-up lipids into already established larger fat droplets. FD, fat droplet; C, cytoplasm; CF, collagen fibrils. Sample taken at 48 hr. Exposure time two months. 22,500 $\times$.

trates the incorporation of *recently-taken-up lipids* into the droplets of intracellularly stored lipids, a process that could not be seen by any other method than autoradiography.

This study is being continued by further elaborating the radioautographic technique toward optimal conditions, including attention to the thickness of the photographic emulsion and the exposure time. The investigation of the liver and the muscle will then proceed. For quantitation of the radioactivity at a given time, to compare the activities in the different tissues and thereby correlate the optical and radiochemical results, we shall complement the electron microscopic autoradiography findings with those of light microscopic autoradiography.

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THE ARRANGEMENT OF THE ELEMENTARY PARTICLES FORMING THE MITOCHONDRIAL MEMBRANE

Theodore N. Tahmisian

PURPOSE AND METHODS

During morphogenesis some organelles are formed *de novo* or synthesized at the site of differentiation. Other organelles are rearranged from existing ones by morphological transformation but with the same function. During spermatogenesis the mitochondrion is transformed to the middle piece of the sperm, maintaining the elementary particles of the mitochondrion. It was reported by us that elementary particles appear in the mitochondrion, in *Helix aspersa*, prior to its deposition at the middle piece.⁽¹⁾

Fernandez-Moran,⁽²⁾ Green et al.,⁽³⁾ and Parsons⁽⁴⁾ were able to show the presence of elementary particles from mitochondrial origin. Fernandez-Moran and Green separated the various parts of the elementary particle and reconstituted them: namely, the head, stem, and pedicel. Parsons was able to show elementary particles in osmotically exploded insect sarcosome, which is a specialized mitochondrion of muscle. An exploded sarcosome showed the heads of the particles pointing out from the main body.

We have shown the orderly appearance and transformation of mitochondrial elementary particles forming the larger part of the middle piece of *Helix aspersa* sperm.

A photomicrograph of a grasshopper spermatocyte mitochondrion fixed under isotonic conditions (Figure 77) indicates that elementary particles form the mitochondrial double membrane, as well as the double membrane of the cristae mitochondriales. This mito-

chondrial section shows that the elementary particles are arranged parallel with the membrane surface. It also suggests that the particles are enmeshed with one another, forming the membrane itself; it also shows that the elementary particles do not protrude from the membrane outward or inward.

This electron photomicrograph, obtained using a Siemens Elmiskop IA at 160,000 × original magnification, shows cross sections of the larger dark circles, 110 Å, the heads; smaller dark circles are again smaller sections of the spherical heads. The 75 Å gray circles are the stems, and the 70 Å by 140 Å rods the pedicels. These particles are present in both the outer and inner membranes, as well as the double membrane of the cristae mitochondriales.

We are certain that the elementary particles are always present in mitochondria and that they are not newly synthesized during differentiation of the mitochondrion to sarcosomes or to the sperm middle piece.

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FIG. 77.—Ultrathin cross section of the grasshopper spermatocyte mitochondrion, $\times 520,000$. Fixed in buffered isotonic OsO_4 solution embedded in Epon and sectioned. H, Head of elementary particle; C, crista mitochondriale showing double membrane; S, cross section of stem; P, pedicel of elementary particle.

A METHOD FOR PREPARING CHROMOSOMES FOR HIGH RESOLUTION ELECTRON MICROSCOPE STUDY

Theodore N. Tahmisian, Rosemarie L. Devine, and Betty J. Wright

PURPOSE AND METHODS

During meiosis and mitosis chromatin undergoes morphological changes that can be observed with the light microscope. We wished to observe the macromolecular nature of meiotic condensed tetrads. For this purpose, a classical preparation of chromosomes for light microscopy was adapted for electron microscopy.

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The grasshopper testis was fixed in hot aceto-orcein (glacial acetic acid and a dye). The acetic acid removes the acid-soluble portion of the cell, leaving the chromosomal DNA. The testis follicle, after it was fixed, was placed on a slide and stained further with diluted aceto-orcein. A cover slip was then placed on the tissue and compressed to spread the chromosomes as seen in Figure 78. In this figure, the eleven tetrads and the X-chromosome are clearly visible. To this point, this method is essentially that used by cytologists for chromosome counts. After noting positions of chromosomal spreads on the slide, the cover slip was removed, and the tetrads left on the microscope slide were dehydrated in ethanol and immersed in propylene

oxide before embedding them in Epon. A wax trough was made about the specimen to contain the Epon at a 2-mm thickness. The epoxy layer was polymerized with heat in the usual manner for electron microscope preparations, and after cooling, it was chipped from the slide lifting the embedded tetrads with it. The desired chips were mounted on aluminum rods and were trimmed for ultramicrotomy.

The specimen was sectioned at 600 Å, stained with uranyl acetate and observed with the aid of the electron microscope. Figure 79 shows a low magnification electron photomicrograph of some of the tetrads. Figure 80, in which 1 mm represents 20 Å, shows certain basic repeating structures which, in this report, are referred to as strands and dots. The double strands are 20 Å apart and the dots are 5 to 8 Å apart. These dots may be the various bases of the DNA chain. Structures found in this type of preparation are to be compared to structures found in conventional electron microscope preparations for the purpose of delineating the skeletal framework of DNA in the latter. Other methods of preparation are also to be divided in the study of chromosomal morphogenesis during spermatogenesis.

FIG. 78.—Aceto-orcein fixed and stained tetraploid chromosomes of *M. differentialis*. Light microscopy; X indicates the X-chromosome. 2500 $\times$.

FIG. 79.—Tetrads fixed in aceto-orcein, sectioned, and stained with uranyl acetate and lead citrate using the new method to obtain chromosomes. 9750 $\times$.

FIG. 80.—A high resolution electron photomicrograph showing DNA helixes and possibly 5-8 Å bases. Arrows indicate 20 Å DNA helixes. 520,000 $\times$.

THE INVESTIGATION OF RADIATION-INDUCED CHROMATID GAPS BY THE ELECTRON MICROSCOPE AND A SCANNING ELECTRON ANALYZING MICROSCOPE

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PURPOSE AND METHODS

The radiation-induced chromosome aberration is one expression of radiation injury in the living cell. There are several types of aberration, but one that characterizes late radiation injury is the 1- or 2-hit aberration,

analyzing microscope.^(1, 2) The latter instrument features a fourth dimension (length, area, volume and electron absorption) in specimen information by detecting unique energy losses that result from characteristic electron absorptions in the specimen.

FIG. 81.—Electron micrograph (RCA) of a subtelocentric chromosome of pig kidney cell in tissue culture 4 yr after irradiation showing “gaps” (G), fibrils (F), and primary fiber (PF) on chromatid arms.

involving all the chromatin threads. A proportion of breaks reconstitute and are capable of further replication. On examination with the phase microscope, they appear as secondary constrictions but are very pale at this locus, giving the impression that their chromatids are broken (“gaps”). Since information on the molecular structure in these areas is limited, we undertook the examination of the chromosome aberrations in irradiated pig kidney cultures, on unsectioned and unstained chromosomes, using the conventional electron microscope (RCA) and a new scanning electron-energy

PROGRESS REPORT

Cells showing radiation-induced chromosome aberrations used for this study were derived from a pig kidney cell strain which was irradiated with 500 R several years ago. Because it was necessary to have a high mitotic index to ensure 1–10 metaphase spreads on a grid, it was necessary to synchronize cells mitotically by temperature shock and Colcemid. Stock cultures, during log phase of growth, were trypsinized and reseeded into fresh culture flasks. After subcultivation, these cells were incubated at 37°C for 24 hr, then

FIG. 82.—A scanning electron analyzing micrograph of pig kidney chromosomes showing different density levels as a result of a specific energy loss sustained by electrons passing through the images.

were transferred to 4°C for a period of 4–24 hr, and after this cold treatment were incubated again at 37°C for 24 hr. During this last period, they were treated with 2 µg/ml Colcemid to obtain cells in arrested metaphase 4 to 6 hr before fixing them. (Under these conditions the mitotic index was between 60–85%.) After treatment cells were removed from the glass surface by trypsinization, washed several times with warm isotonic Hank's balanced salt solution, and then were treated with hypotonic Hank's solution (1:4) for 8 to 10 min. After removing the hypotonic solution, the cells were fixed (3:1) in two changes of methanol-acetic acid for two 10-min periods. It was found that the cells *clumped* in this fixative, and metaphase spreads on the carbon-coated EM grids were poor; therefore, this fixative was centrifuged and decanted, and a 45% aqueous acetic acid solution was added to break up the clumps. A microdrop of this suspension was placed on the grid, or the grid was lowered over a microdrop contained in a well, permitting the grid to touch the drop so it would adsorb the solution containing the cells onto the membrane. In both cases, the grids were permitted to air dry. The cells were not stained prior to observation. Before each grid was placed in an electron microscope, it was examined through a phase microscope to ensure well-spread chromosomes for the electron microscope study. They were recorded by microphotography for later comparative identification.

Figure 81 shows the chromosome with "gaps" in the

chromatids through the conventional electron microscope (RCA). These areas (G) contain less chromatin material, suggesting an incomplete replication of secondary chromatin strands. There are many minute fibrils (F) projecting from the chromatid arms. Since they are numerous, it is unlikely that they are spindle fibers, but may be similar in structure to the lampbrush fibrils.⁽³⁾ At intervals along the chromosome, there are recognizable primary fibers (PF) which are about 500 Å wide,⁽⁴⁾ and they are within the size range of the Watson-Crick DNA helix when surrounded by an uncoiled nonhistone protein coat.⁽⁵⁾ Because of the thickness of the chromosome, it is difficult to tell if there is a helical arrangement, but this is suggested.

CONCLUSION

Information revealed by the conventional electron microscope has not yet been discerned with the scanning electron microscope. This microscope is so designed, however, that it can selectively localize specific elements, such as phosphorous, on the basis of specific energy losses sustained by electrons as they impinge on the element in question. Because of the high phosphorous content of DNA, this method should serve to identify DNA in the chromosome. The chromosomes in the photograph (Figure 82) show different density levels suggesting that those chromosomes with the greatest density have some element in a higher concentration than the least dense chromosomes.

We wish to acknowledge the helpful assistance of Arlene Zadylak in photographing the chromosomes with the RCA electron microscope and in the identification of ultrastructure.

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INDUCTION OF ULTRASTRUCTURAL LESIONS IN RAT ADRENAL CORTEX BY 3-ACETILPYRIDINE

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PURPOSE AND METHODS

The known induction of gross necrotic lesions of the medulla of the adrenal gland by 3-acetylpyridine⁽¹⁾ administered intraperitoneally parallels the autoradiographic evidence of accumulation of this nicotinamide analog in this organ.⁽²⁾ Despite the recognition that nicotinamide is required for steroidogenesis, no previous data are reported that demonstrate a correlation of the disrupted biochemical reactions caused by this vitamin analog with ultrastructural changes of the adrenal cortex. Previous studies of the physiological action of this antimetabolite⁽³⁾ showed that the compound is metabolized to nicotinic acid and the pyridone of *N*-methylnicotinamide. This rapid conversion of the analog into the normal metabolite thus limits the action time of 3-acetylpyridine. The development of gross lesions of the adrenal gland after the conversion and elimination of the abnormal coenzyme would be expected to be preceded by initial ultrastructural modifications. This study was focused on these early intracellular changes which could be investigated by an electron microscopic study of the adrenal gland of rats challenged with 3-acetylpyridine.

Unmated, mature SD/Anl (Anl 66) rats, varying from 160 to 210 g were paired as experimental and control animals by sex, age, and weight. All of the animals were starved for 24 hr before they were injected. The experimental animals received intraperitoneal injections of 20 mg/kg of 3-acetylpyridine (methyl 3-pyridylketone, obtained from Eastman) in physiological saline solution. Control animals were injected with physiological saline. The animals were killed by cervical dislocation. The adrenal glands were immediately excised, minced and fixed in phosphate-buffered 2% glutaraldehyde fixative containing 5% sucrose for 24 hr and then washed in two changes of phosphate buffer for 8 hr. The fixed tissue was then post-fixed for 2 hr in buffered osmium tetroxide. After dehydration, the tissues were given a final wash in 3 changes of propylene oxide and embedded in Epon-Araldite plastic.

Thick sections (4–5 μ) for light microscopy were cut with glass knives and examined without staining with a phase contrast microscope. These thick sections permitted the identification of zonation characteristics and the selection of areas for ultrathin sections for elec-

tron microscopy. Silver-gray sections (600–800 Å) were cut either on an LKB or Cambridge Ultramicrotome with glass or diamond knives. Grids were stained in saturated uranyl acetate for 2 hr and then in Reynold's lead stain⁽⁴⁾ for 15 min. These doubly stained grids were then thoroughly rinsed in 0.02 *M* NaOH. Electron micrographs were obtained by using RCA Electron Microscopes.

PROGRESS REPORT

The mitochondria of the adrenal cortex are different from the mitochondria of most mammalian cells in that they have a matrix filled with tubular or vesicular structures instead of laminar cristae. Mitochondria of the *zona glomerulosa* display elongate tubular forms, whereas the mitochondria of the *zona fasciculata* and the *zona reticularis* appear more vesiculated. The cells of the adrenal cortex of the rat are almost filled with these spherical or ovoid mitochondria of various sizes up to 2.5 μ in diameter. Multiple, electron-dense lipid droplets are present in most of the cells of the normal control animals. These accumulations of steroids are sharply delineated from the remaining cytoplasm and, in turn, are surrounded by a halo of ribosomes.

A proliferation of lysosome-like bodies in the endothelial cells during the first few hours after 3-acetylpyridine injection precedes their appearance in the secretory cells of the adrenal cortex. Progressive degenerative changes occur within these secretory cells and by 24 hr after injection, several large, darkly-stained masses appear proximal to normal appearing mitochondria. Many of these distorted ovoid bodies have vesiculated internal structures similar to the tubular cristae of the normal adrenal cortex mitochondria. Within these abnormal, darkly-stained structures are numerous lipid-like accumulations of various sizes. Large, whorled bodies appear inside some otherwise normal appearing mitochondria. Similar dark masses have been observed in the mitochondria of ACTH-treated animals.⁽⁵⁾ The progressive deterioration, indicated by the abnormal mitochondria, is further accompanied by a decrease in the electron density of the lipid droplets. Although there is a general decrease in the frequency of these lipid bodies, there is neither obvious crenation nor indentation of the lipid droplets, as is frequently seen in ACTH-treated animals.

The ultrastructural modifications induced in the

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adrenal cortex of the rat challenged with 3-acetylpyridine are reminiscent of, but different from changes induced in ACTH-treated or stressed animals.⁽⁶⁾

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DETERMINATION OF GLUTARALDEHYDE IN FIXATIVE SOLUTIONS

Norman A. Frigerio and Michael J. Shaw

PURPOSE AND METHODS

As part of our continued interest in tissue fixatives, we applied our iodometric method for the determination of glutaraldehyde (GA)⁽¹⁾ to examination of the

TABLE 18. GRAVIMETRIC ANALYSIS OF GLUTARALDEHYDE IN PHOSPHATE-SUCROSE BUFFER AS A FUNCTION OF CONCENTRATION

Volume of 6.24% GA in phosphate-sucrose buffer added, ml	Volume of water added, ml	Concentration of glutaraldehyde found, % (w/w)	Concentration of GA normalized to undiluted value, % (w/w)
1.0	0.0	6.234	6.234
1.0	0.0	6.236	6.236
1.0	1.0	3.121	6.241
1.0	1.0	3.120	6.240
1.0	5.0	1.040	6.237
1.0	5.0	1.041	6.246
			6.239 $\pm$ 0.003

TABLE 19. GRAVIMETRIC ANALYSIS OF GLUTARALDEHYDE IN CACODYLATE BUFFER AS A FUNCTION OF CONCENTRATION

Volume of 6.27% GA in cacodylate buffer added, ml	Volume of water added, ml	Concentration of glutaraldehyde found, % (w/w)	Concentration of GA normalized to undiluted value, % (w/w)
1.0	0.0	6.239	6.239
1.0	0.0	6.247	6.247
1.0	1.0	3.137	6.274
1.0	1.0	3.140	6.281
1.0	5.0	1.046	6.274
1.0	5.0	1.042	6.254
			6.262 $\pm$.015

storage properties of GA in three common fixative solutions.

PROGRESS REPORT

GA was determined as a function of concentration in (a) $\text{Na}_2\text{HPO}_4\text{-KH}_2\text{PO}_4$ buffer, 0.02 *M* in phosphate, pH 6.85; (b) $\text{Na}_2\text{HPO}_4\text{-KH}_2\text{PO}_4$ buffer made 6% w/v in sucrose; and (c) 0.0275 *M* sodium cacodylate buffer (pH 7.25). Results of tests (b) and (c) are presented in Tables 18 and 19.

A variation of reaction time between 5 and 30 min had no significant effect (i.e., <0.001%) on the results obtained, using GA concentrations between 0.6 and 6.0%, when either water, 0.02 *M* phosphate buffer (with or without sucrose), or 0.0275 *M* cacodylate buffer was used.

To test the effect of time and temperature on deterioration, four 2.6% solutions of GA in water, phosphate buffer with sucrose (6%), phosphate buffer without sucrose, and cacodylate buffer were prepared using distilled water of conductivity grade (i.e., $\leq 300,000 \Omega$ specific resistance at 1°C). Each GA solution was divided into two parts, one stored at 25°C, and the other at 1°C, in glass stoppered volumetric flasks. Results are presented in Table 20.

The presence of sucrose had no effect on the determination of GA, nor did it significantly influence the storage properties of GA at these concentrations. A slight increase in apparent aldehyde content as a function of storage time was noted in water. This increase may be attributed to dissociation of polymeric glutaraldehyde, formed from the acid-catalyzed* elimination of water between successive molecules of glutaraldehyde hydrate, in much the same manner as formaldehyde hydrate.⁽²⁾

* pH of 50% GA was 3.2.

Previously, we reported⁽¹⁾ that a 2.6% aqueous solution of GA stored at 24°C dropped by 0.16% within 4 days. We now find that a 2.6% aqueous GA solution does not change significantly even in 7 days. This difference is attributed to the fact that less vigorously purified water was used in the former experiment. Certainly dialdehydes are sensitive to heat, oxygen, light, and base, especially in the presence of traces of transition metals which are often found in water.⁽³⁾ Thus, one can never predict the stability of any given dialdehyde solution, and check determinations should be made if reproducible results are to be obtained.

A fresh sample of nominal 50% (w/w) solution of GA was titrated with 0.01 *M* NaOH as a function of storage time. After storage for 41 days at 1°C the acid concentration increased from 1.107×10^{-5} mole

equivalents of acid/g to 1.525×10^{-5} mole equivalents of acid/g. After one year, the acid concentration was 1.638×10^{-5} mole equivalents of acid/g, and the GA content had diminished from 48.31 to 45.85% (w/w). Thus, 48.3 moles of GA were destroyed per mole of hydronium ion generated. It is known that formaldehyde and acetaldehyde rapidly polymerize, via acid catalysis, to trimeric forms:

These are relatively insoluble, not easily oxidized, and show no carbonyl reactions. At equilibrium, conversion is about 95% complete.⁽²⁾ We suggest that the same type of reaction occurs with GA. This would account for the low ratio of acid production to aldehyde disappearance, the observed production of an insoluble polymeric product in concentrated solutions and, by dilution reversal, the increase of aldehyde concentration on standing in diluted solutions (Table 20).

CONCLUSIONS

Measurement of the amount of unreacted bisulfite has afforded a simple, rapid, and precise method for the determination of glutaraldehyde, in aqueous solution and in common tissue fixative solutions. Quantitative analyses have indicated that periodic check determinations of GA should be made if reproducible tissue fixation is to be expected.

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TABLE 20. GRAVIMETRIC ANALYSIS OF GLUTARALDEHYDE AS A FUNCTION OF STORAGE TIME, DILUENT, AND TEMPERATURE

Solution	Stored at 25° C		Stored at 1° C	
	Storage time, hr	GA Conc., % (w/w)	Storage time, hr	GA Conc., % (w/w)
2.6% GA in water	0.3	2.629	0.3	2.629
	4.0	2.636	24.0	2.626
	8.0	2.656	49.0	2.631
	22.0	2.676	96.0	2.634
	49.0	2.671	168.0	2.621
	168.0	2.684		
2.4% GA in phosphate sucrose buffer	0.5	2.481	0.5	2.481
	7.0	2.503	29.0	2.482
	27.0	2.482	49.0	2.510
	48.0	2.486	100.0	2.566
2.5% GA in phosphate buffer	0.75	2.570	0.75	2.570
	29.00	2.565	30.00	2.568
3.1% GA in cacodylate buffer	0.5	3.117	0.5	3.117
	6.0	3.111	25.0	3.091
	25.0	3.064	75.0	3.110
	75.0	3.045		

THE PHOTOINHIBITION OF COLEOPTILE GROWTH AND ITS BEARING ON PHOTOTROPISM

Solon A. Gordon and Gerald A. Brown

PURPOSE AND METHODS

Growth of the young oat shoot, the coleoptile, is limited by the hormone, auxin. This hormone is produced in the tip of the shoot, transported downward, and stoichiometrically controls the rate of elongation of the tissues beneath the tip. We have postulated^(1, 2) that phototropism derives from an impairment by light of the downward (basipetal) transport of auxin—more explicitly, that unilateral irradiation of a shoot at first-positive radiant densities causes a lateral difference in the ability of the tissues of the shoot to transport auxin basipetally. The resultant lateral difference in concentration of the hormone gives rise to the difference in growth of proximal and distal tissues that is manifest as curvature. If this hypothesis is correct, one would expect that (a) the same energies given *equilaterally* would curtail the extension of the coleoptile; and (b) the kinetics, the exposure-response curves, and the action spectra for the impairment in growth and for phototropism would be similar. To test these expectancies, the effect of equilateral illumination on the elongation of the oat shoot has been investigated.

Etiolated oat coleoptiles were given various equilateral exposures to monochromatic bands of the Argonne Biological Spectrograph. The height of the coleoptile was determined by shadowgraph photography⁽³⁾ immediately before illumination, and at 1 and 2 hr afterwards. As controls, seedlings treated identically but not exposed to the spectrograph beam were run concomitantly in every exposure sequence.

PROGRESS REPORT

Irradiation of the 3-day-old, dark-grown, oat seedling at the 445-nm locus of the spectrograph caused a reduction in length of the coleoptile. The impairment of growth was maximum at 1 to 1½ hr after irradiation and then decreased, a response consistent with the kinetics of the phototropic reaction.⁽⁴⁾ Figure 83 shows the exposure-response curve at 445 nm. The impairment of growth increases with radiant energy, reaches

a maximum at about 50 ergs·cm⁻², and decreases to zero with energies on the order of 1 kiloerg·cm⁻². This unique exposure-response curve matches almost exactly that of phototropic curvature at the same wavelength.⁽⁵⁾

The spectral dependence of first-positive phototropism has been investigated in several laboratories. Two of the action spectra^(6, 7) are in approximate agreement. These curves show phototropic response only in the blue and near-ultraviolet spectral regions, with band maxima at 370, 445, and 475 nm. Response to first-positive energies disappears at wavelengths greater than 525 nm. Therefore, the quanta equivalent to 25 ergs·cm⁻² at 445 nm (5.6×10^{-12} quanta·cm⁻²) were given unilaterally to *Avena* coleoptiles placed at various wavelength positions of the spectrograph. The seedlings were rotated for exactly one revolution during exposure. Growth rates of the coleoptiles for the first hour after exposure at the various wavelength loci are shown in Figure 84. The data show a retardation of growth in the region of 370 to 475 nm. Wavelengths above 500 nm were ineffective at the incident energies used. Thus the spectral efficiencies for the

FIG. 83.—Growth rates of the *Avena* shoot after equilateral irradiation with monochromatic light, 445 nm in wavelength.

FIG. 84.—Action spectrum for the photoimpairment of growth of the *Avena* shoot.

photoinhibition of elongation correspond broadly to the previously described action spectra for phototropism.

CONCLUSIONS

Equilateral irradiation with monochromatic blue light causes a reduction in elongation of the etiolated coleoptile. The kinetics of this impairment and its exposure-response relationship match those of first-positive phototropism. An action spectrum for this

photoinduction of growth inhibition is similar to the relative spectral response for phototropism. These observations support strongly the hypothesis that the primary response to unilateral irradiation at first-positive radiant densities is a differential inhibition in the rates at which the hormone is transported basipetally.

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HORMONAL RELATIONS IN THE PHOTOTROPIC RESPONSE. V. PHOTODINHIBITION OF AUXIN TRANSPORT AND ITS BEARING ON PHOTOTROPISM

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PURPOSE AND METHODS

We have proposed⁽¹⁾ that the primary hormonal phenomenon in phototropism is a light-induced differential impairment in the basipetal movement of auxin. Supporting this hypothesis are the unique, almost identical, dosage-response curves for phototropism and for the photoimpairment in basipetal transport.⁽²⁾ In this report, we will show that the action spectrum (340-730 nm) for the transport inhibition corresponds approximately to that for phototropism.

The Argonne spectrograph, powered by a 240 W tungsten ribbon-filament lamp, was used as the source of monochromatic light. The emission from this assembly was stable and provided a smooth continuum cross the wavelength range examined. Etiolated oat seedlings were placed on a rotating horizontal disc on the focal curve of the spectrograph and irradiated

unilaterally. The rotation rate was adjusted so that each seedling underwent one revolution during the exposure. Immediately after irradiation, C^{14} -indoleacetic acid (IAA) in aqueous solution was applied to the apex of the coleoptile. One hour after IAA application the shoot was severed from the seed and washed with distilled water to remove residual IAA from the surface. The shoot was then divided into three parts: tip, 1 mm; the next or "mid," 4 mm; and the remaining basal portion. Radioactivity was determined in each segment by scintillation counting.

PROGRESS REPORT

Figure 85 shows the exposure-response curves for parallel experiments on the effect of blue light (445 nm) on phototropism and the inhibition of auxin transport. The degree of curvature increases with increasing illuminance, reaching a plateau at ca. 40-80 ergs $\cdot$ cm⁻².

FIG. 85.—Dose-response curves for phototropism and for the inhibition of ^{14}C -IAA transport with blue light, 445 nm. Solid line, phototropism of the *Avena* coleoptile; broken line, relative transport inhibition.

FIG. 86.—Action spectra of phototropism and of the inhibition of ^{14}C -IAA transport. Incident energy 1.34×10^{13} quanta·cm $^{-2}$. Solid line, phototropism of the *Avena* coleoptile; broken line, relative transport inhibition.

With further increase in radiant densities, the curvatures decrease. The transport inhibition increases and decreases with the curvature response. The maximum transport inhibitions are between 50 and 100 ergs·cm $^{-2}$.

For the action spectrum of transport inhibition, a quantum equivalent to 60 ergs·cm $^{-2}$ at 445 nm (1.3×10^{13} quanta·cm $^{-2}$) was used for each of the wavelengths tested. Figure 86 shows the relative spectral

efficiencies (340–730 nm) for transport inhibition and phototropic curvature. The two phenomena were tested simultaneously. Here again, the action spectrum for transport inhibition parallels roughly that for phototropism. The inhibition maxima are at 365, 430–445, and 475 nm; minima occur at 340, 425, and 465 nm. These inflections are similar to those observed for first-positive phototropism by Curry and Thimann and Shropshire (cf. Reference 3).

CONCLUSIONS

We find that blue light at the radiant densities of first-positive phototropism inhibits the basipetal transport of auxin. The exposure-response for transport inhibition matches that for the phototropic response. The action spectrum for transport inhibition also resembles that for phototropism. These findings support the hypothesis that phototropism after unilateral irradiation at first-positive radiant densities is a consequence of a lateral difference between the ability of the irradiated and shaded tissues to transport auxin basipetally.

THE EFFECT OF TEMPERATURE AND GIBBERELIC ACID ON FLOWERING IN OENOTHERA

William Chorney and Solon A. Gordon

PURPOSE AND METHODS

Under natural conditions *Oenothera* is a biennial. It normally flowers on long days after winter vernalization. With some long-day, cold-requiring rosette species, gibberellins can substitute for the cold requirement to induce flowering. Picard was unable to obtain flowering in *Oenothera biennis* L. by prolonged treatment with gibberellic acid after a period of vernalization.⁽¹⁾ Chouard, using a variety of *Oenothera lamarkiana*, from the forest of Fontainebleu, was successful in promoting flowering by application of gibberellic acid.⁽²⁾ This study was undertaken to determine whether temperature and gibberellic acid treatment could modify the flowering response in *Oenothera* under greenhouse conditions.

Seeds of *Oenothera lamarkiana* (cv. Japan) were sown on October 5, 1966; the seedlings were grown in a greenhouse under the natural daylength. On November 2, after the photoperiod was changed to 18 hr, the experimental treatments were initiated (Table 21). Plants were sprayed with gibberellic acid (GA₃), 100 mg/l, dissolved in 0.1% Tween-20, or with 0.1% Tween-20 alone. The temperature treatment was initiated two months after sowing, by subjecting plants to 5° or 23° for 21 or 42 days. One month prior to the start of the temperature treatment, and again 3 days later, the plants were sprayed. On termination of the temperature treatment the plants were sprayed 4 times, twice weekly at 3- and 4-day intervals. Growth of the plants took place on the greenhouse bench on an 18 hr photoperiod. Final observations on growth and flowering were taken 66 days after termination of the temperature treatment. There were 14 replicates in each of

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the 16 treatments, with the bench locations of the plants being randomized.

PROGRESS REPORT

Since the growth of the plants from the 21- and 42-day temperature treatments took place at different times, comparisons between these two treatments were not made. Plants supplied with GA₃ after the temperature treatment bolted and flowered, irrespective of the temperature and the duration of the exposure. All plants that did not receive the post GA₃ spray remained in the rosette form for the entire experimental period.

An enhancement of flower initiation is indicated by a reduction in the number of nodes to the first flowering node. The first flowering node was observed at 4 and 14 nodes lower when plants were subjected to 5° for 21 and 42 days, respectively, (Table 22). Floral initiation occurred about five nodes lower in plants treated with GA₃ prior to the temperature treatment, irrespec-

TABLE 21. EXPERIMENTAL TREATMENTS TESTED. DURATION OF TEMPERATURE EXPOSURE 21 AND 42 DAYS

GA ₃ treatment prior	Temperature, °C	GA ₃ treatment post
+	5	+
-	5	+
+	5	-
-	5	-
+	23	+
-	23	+
+	23	-
-	23	-

+ Sprayed with gibberellic acid; - Sprayed with Tween-20.

tive of the temperature and length of the treatment. Spraying *Oenothera* with GA₃ after exposure to low temperature will hasten floral initiation. GA₃ applied before subjecting plants to low temperature appears to act independently in enhancing floral initiation.

Plants that were exposed for 21 days to the 5° temperature produced the first open flower 9 days sooner than those plants that received the 23° temperature. GA₃ applied prior to the 21-day, 5° temperature treatment did not alter the number of days required to obtain the first open flower. However, GA₃ applied prior to the 21-day, 23° temperature treatment shortened by 7 days the time to first mature flower. No ef-

TABLE 22. EFFECT OF GIBBERELIC ACID AND TEMPERATURE ON THE NUMBER OF NODES TO FIRST FLOWER EMERGENCE

GA ₃ prior	Temperature, °C	GA ₃ post	Temperature exposure, days	
			21	42
+	5	+	30.8	29.5
-	5	+	36.5	33.3
+	23	+	34.1	42.5
-	23	+	40.6	47.1
			N 14	14
			M.S.E. 6.44	7.52

All means within a column are different at odds of 99:1 or greater.

TABLE 23. EFFECT OF GIBBERELIC ACID ON THE INTERVAL (DAYS) BETWEEN END OF TEMPERATURE TREATMENT AND INCIDENCE OF FIRST OPEN FLOWER

GA ₃ prior	Temperature, °C	GA ₃ post	Temperature exposure, days	
			21	42
+	5	+	51.1 _a	57.3 _c
-	5	+	51.9 _a	59.1 _{ab}
+	23	+	54.1 _a	58.1 _{bc}
-	23	+	60.8	60.2 _a
			N 12	14
			M.S.E. 6.44	2.56

Columnar means followed by a common subscript do not differ at odds of 99:1 or greater.

TABLE 24. EFFECTS OF GIBBERELIC ACID AND TEMPERATURE ON STEM LENGTH

GA ₃ prior	Temperature, °C	GA ₃ post	Temperature exposure, days	
			21	42
+	5	+	76.0 _{ab}	65.1 _a
-	5	+	79.6 _a	63.9 _a
+	23	+	72.4 _{bc}	72.2
-	23	+	70.3 _c	64.0 _a
			N 14	14
			M.S.E. 46.27	58.70

Columnar means followed by a common subscript do not differ at odds of 19:1 or greater.

fect of temperature on flower maturation was observed in plants that were exposed for 42 days, but prior GA₃ application shortened by 2 days the time required to obtain the first mature flower at both temperatures (Table 23).

Prior GA₃ application was ineffective in modifying stem length in the 21-day temperature treated plants (Table 24). However, the 5° plants showed a 9-cm increase in stem length. Stem lengths were not different in plants that were exposed for 42 days to the two temperatures, but prior application of GA₃ to the 42-day, 23° plants resulted in a 7-cm stem length increase.

CONCLUSIONS

Oenothera lamarckiana (cv. Japan) will initiate and produce mature flowers under greenhouse conditions on an 18-hr photoperiod after treatment with gibberellic acid. When gibberellic acid is applied after cold temperature exposure, the growth substance interacts with the cold temperature treatment to hasten flowering. Application of gibberellic acid prior to the temperature treatment acts independently of temperature in modifying the flowering process.

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ACTION SPECTRUM FOR FLORAL BUD OPENING IN WHITE PHARBITIS

Toshio Yamaki and Solon A. Gordon

PURPOSE AND METHODS

In a previous report⁽¹⁾ we described the inhibition by light of the opening of flower buds of *Pharbitis nil* cv. violet. Its action spectrum differed from that of *Oenothera*. The relative spectral efficiencies and photo-reversibilities for *Pharbitis* were those of a phytochrome-mediated reaction. We became curious as to whether the white-flowered cultivar resembled the violet in this spectral response. Accordingly, spectral efficiencies for the photoinhibition of bud opening were determined by procedures similar to those used for the violet form.⁽¹⁾

PROGRESS REPORT

Mature plants bearing unopened flower buds were given a 5-day prephasing in a photoperiod room and then exposed to various monochromatic beams on the focal curve of the spectrograph. The various stages of floral opening, identified by numerical values, were scored at each wavelength locus and used to calculate inhibition indices. These indices per unit energy absorbed were plotted as a function of wavelength (Figure 87).

CONCLUSIONS

We conclude that the action spectrum for the photoinhibition of floral bud unfolding in the white cultivar is identical to that possessed by the violet—both have a major maximum band at 700 nm with a sharp drop in the far-red, some slight activity in the blue spectral

FIG. 87—Relative spectral efficiencies for the inhibition by light of flower bud opening in *Pharbitis nil* cv. white.

region, and the indication of a shoulder at about 550–560 nm. It is thus probable that the photomorphogenic pigment for the inhibition of flower bud unfolding in the white cultivar is also phytochrome.

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FURTHER EXPERIMENTS ON THE ACTION SPECTRUM AND PIGMENTS FOR THE PHOTONHIBITION OF FLOWER BUD OPENING IN OENOTHERA

Toshio Yamaki, Solon A. Gordon, and William Chorney

PURPOSE AND METHODS

Mature flower buds of many of the *Oenothera* normally open within several hours after dusk. Opening of the flower is prevented by exposure of the bud to light. In preliminary experiments with field cuttings of *O. biennis*,⁽¹⁾ we began to delineate the action spectrum for this photoinhibition. Significant inhibition for lower opening occurred at wavelengths between 410 and 520 nm, with bands at 480 to 490, at about 510 to 515, and at 535 to 540 nm. There were consistent minima between these bands.

The field cuttings varied widely in photoresponsivity. This variability, and the dependence on field search, made a more uniform stable source of plant material desirable for the spectral studies. Elsewhere in this report, a successful method for growing the biennial as an annual under greenhouse conditions is described.⁽²⁾ Using this technique, additional observations have been made on the action spectrum and on the pigment systems that may be involved in the photoresponse.

PROGRESS REPORT

Flowering plants of *Oenothera lamarckiana* were obtained, using the gibberellin technique,⁽²⁾ except that no cold treatment was used. Plants were moved to a preconditioning room at 10:00 a.m. 2 or 3 days before experimental use. This room was maintained at 33°C during the light phase and 25°C during the dark; the light cycle began at 5:00 a.m. and ended at 5:00 p.m. Illuminance was supplied by luminaires containing 96T8 Standard Cool fluorescent tubes. The illuminance at terminal bud level was about 1200 fc. This preconditioning shifted the period of bud opening to times more convenient experimentally. At 4:40 p.m., the plants were placed along the spectrograph focal curve under banks of Standard Cool fluorescent tubes (about 800 fc at plant level). Irradiation at the various wavelength loci began at 4:50 p.m., while the plants were still under white light, in order to adjust the positions of the flower buds in the monochromatic beams. The white light was extinguished at 5:00 p.m. Radiant densities at each wavelength were calculated from the time at which the exposure to white light was ended. Control plants were also exposed to the white light at the spectrograph focal curve, but at 5:00 p.m. these plants were placed in light-tight chambers in the same room.

The quantitative definition of the various stages of floral closure used before⁽¹⁾ could not be applied in these experiments because of the rapidity with which the buds on intact plants opened. (The rate of bud opening on the field cuttings used previously was materially slower. Furthermore, bud-bearing stalks cut from the root stock and placed in water opened more slowly than did those on intact plants. Also, flower buds excised at the stem surface and placed in water opened still more slowly.) Therefore, the number of

buds completely open at each wavelength was determined every five minutes after initiation of exposure to monochromatic light. The time at which 50% of the flower buds opened was estimated by interpolation of the response curve and used for calculation of the inhibition index.⁽¹⁾ These inhibition indices were divided by the total energy incident to the buds during the time for a 50% response. The resultant inhibition efficiencies are plotted against wavelength in Figure 88.

A well-defined major band occurs at 675 nm. Major bands also appear at 385 nm and between 440 and 480 nm. There appears to be a shoulder at 420 nm, and some structure between 440 and 480 nm. The bands at 505 and 535 nm are small, but appear consistently.

Pigments from the mature sepals, the site of the photomorphogenic sensitivity (unpublished data of Yamaki and Saito), were then extracted as a clue to the photoreceptor. Several extraction procedures were tested, using similarities between the action spectrum and the absorption spectra of the fractions as an index to pigments of interest. The following procedure proved useful. Gross pigment mixtures were extracted from the sepals with 80% ethanol. Absorption spectra of this ethanolic extract showed bands at 505 and 535 nm, as well as the major bands in the red and blue. Accordingly, the pigments were transferred from the alcohol into petroleum ether (30°–60° b.p.). The ether was washed with water to remove flavonols, dried with anhydrous sodium sulfate, concentrated by air draft, and fractionated on silica gel columns, using 1 to 15% isopropanol in petroleum ether as a developing agent.

Silica gel fractionation separated about 15 bands, four or five of which possessed carotenoid-type absorption spectra. One band, bluish-green at low concentrations in petroleum ether and grey-green at higher concentrations, possessed well-defined absorption bands in the green as well as major bands in the red and blue spectral regions. This pigment was taken up with 1% isopropanol in petroleum ether and resolved on a column of alumina (Brockmann grade II). The same solvent was used for elution. Two bands were resolved. One was purified by a second passage through silica gel (Davison, 41 Å pore diameter) using 1% ethanol in petroleum ether. The absorption spectrum of this pigment in ether is shown in Figure 89. It strongly resembles those of the tetrapyrroles. The absorption spectrum of pheophytin *a*⁽³⁾ is also shown in the figure. The similarities between the two spectra are apparent. However, we hesitate to suggest that the pigment isolated does function photomorphogenically in the inhibition of bud opening; the action spectrum shows a broad, structured band between 420 and 480 nm, whereas the isolated pigment has a relatively narrow

FIG. 88—Inhibition indices per unit radiant density for flower bud opening in *Oenothera lamarckiana*. Each datum represents the mean of 4 to 10 experiments at different times, with 10 to 15 buds tested in each experiment.

FIG. 89—A, Absorption spectrum of pigment isolated from sepals of flower buds of *O. lamarkiana* (broken line) at 3× concentration; B, absorption spectrum of purified pheophytin *a*.⁽³⁾

band near 400 nm. The action spectrum also has a band in the near ultraviolet, a band at which no more than a trace shoulder occurs in the absorption spectrum. It is possible that these discrepancies might derive from an *in vivo* complex of the pigment from which the chromophore is separated by organic solvent. Alternatively, the pigment isolated from *Oenothera* (tentatively pheophytin *a*) might have originated

as a degradation product of chlorophyll *a* during the isolation. We can infer, however, that the action spectrum is consistent with the function of a porphyrin as the primary chromophore. Further work on the resolution of these *Oenothera* pigments is continuing.

CONCLUSIONS

A study of the action spectrum for the photoinhibition of flower bud opening in *Oenothera lamarkiana* has been completed. Major bands occur at 675 nm and between 420 and 475 nm. An intermediate band occurs at about 385 nm, with two lesser bands at 505 and 535 nm. A pigment whose absorption bands match some but not all of the action spectrum characteristics has been isolated from the sepals of the flower bud and chromatographically resolved. Its absorption spectrum is virtually identical to that of pheophytin *a*.

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GROWTH AND DEVELOPMENT OF PLANTS IN COMPENSATED GRAVITATIONAL, MAGNETIC, AND ELECTRICAL FIELDS

GRAVITY COMPENSATION AND INTRACELLULAR PERIODICITIES IN X-IRRADIATED VICIA SEEDLINGS

Solon A. Gordon and Evelyn M. Buess

PURPOSE

As an index of the influence of gravity on radiation response, we are examining the effect of gravity compensation by clinostat on nuclear components and behavior. A previous report⁽¹⁾ described the chromosome aberrations induced by X radiation in root tips of the broad-bean seedling. Roots of compensated and vertically rotated (control) plants were analyzed cytologically for 54 hr following exposure of the organs to 100 R. There was no material effect of gravity compensation on the total number of chromatid aberrations of the various types scored (breaks, isochromatids, exchanges, and aberrant cell frequency). However, a consistent decrease in these aberration indices was found in compensated organs 6 hr after irradiation. This was followed by a consistent rise in frequency over the vertically-rotated controls at 12 hr. There was also a shift in the periodicity of the number of cells in metaphase per root tip associated with compensation. To investigate further this indication that gravity can influence circadian periodicity, "squash" preparations have been examined by microspectrophotometry⁽²⁾ for variations in Feulgen dye bound to interphase nuclei, for alterations in the incidence of micronuclei, and for changes in nuclear volume.

PROGRESS REPORT

Seeds of *Vicia faba* were dusted with Ceresan-M and soaked in water at 4 C for 24 hr. After removal of the seed coat, the seeds were inserted into "dolls" of filter paper⁽³⁾ and placed in cylindrical jars, the jars being clamped to clinostats of the serpentine type. Two axes of rotation were employed—horizontal and vertical. The plants were grown in the dark at 24 C and exposed *in situ* to 100 R X radiation (HVL 1.6 mm Cu) 8 days after planting. Positions of the seedlings on the clinostats were chosen on the basis of previous phantom dosimetry so that exposures differed from one another by no more than 5%. Rotational motion was maintained in the 8 days before, during, and after X irradiation. At various times after irradiation three jars

from each treatment were taken for analysis. Seedlings were not exposed to light after planting except at the time of X irradiation, when green light at an irradiance level barely enough to enable manipulation was used. Only secondary roots were taken for analysis. These were treated with colchicine for 2 hr. The tip of each root was then placed in Ford's fixative, stained with Feulgen reagent,⁽⁴⁾ and its apical 2.0 mm was mounted permanently as a squash preparation.

For the determination of nuclear volume and absorbancy, horizontal scans were made across the slide at the $\frac{1}{3}$, $\frac{1}{2}$ and $\frac{2}{3}$ intervals of the vertical dimension. At each 1-mm position of the scan, the interphase nucleus closest to the center of the field was measured. Measurements were continued until a total of 20 nuclei were obtained for each root tip. Five root tips were analyzed per experimental treatment. The micronuclei were scored in all interphase cells encountered in $\frac{1}{6}$ vertical scan intervals at 1200 $\times$ magnification.

Table 25 shows the amount of dye bound per interphase nucleus at various times after irradiation. No

TABLE 25. RELATIVE AMOUNTS OF FEULGEN DYE BOUND PER INTERPHASE NUCLEUS IN ROOT-TIP CELLS OF *Vicia faba*. EACH DATUM IS THE MEAN OF 5 ROOTS, 20 NUCLEI PER ROOT

Hours after X irradiation	Compensated	Vertically rotated
0	9.0 $\pm$ 0.29	11.4 $\pm$ 0.35
4	13.6 $\pm$ 0.34	10.9 $\pm$ 0.32
6	8.8 $\pm$ 0.28	11.6 $\pm$ 0.34
8	12.5 $\pm$ 0.33	10.9 $\pm$ 0.26
12	12.6 $\pm$ 0.34	13.2 $\pm$ 0.37
18	12.6 $\pm$ 0.57	11.5 $\pm$ 0.33
24	14.2 $\pm$ 0.38	11.3 $\pm$ 0.38
30	9.0 $\pm$ 0.18	9.7 $\pm$ 0.34
36	11.9 $\pm$ 0.38	12.0 $\pm$ 0.30
42	9.1 $\pm$ 0.23	10.4 $\pm$ 0.25
48	10.0 $\pm$ 0.24	8.4 $\pm$ 0.23
54	11.7 $\pm$ 0.36	9.9 $\pm$ 0.18
Treatment mean	11.2 $\pm$ 0.57	10.9 $\pm$ 0.36

clear pattern of change with time is apparent. Neither was there a material effect of the gravitational treatment on what is presumed to be an index of DNA content; the means for the two gravity-treatment populations are not statistically different.

This absence of a defined periodicity and lack of an effect of gravity on apparent DNA content was surprising in view of the cyclic changes in nuclear volume with both time and treatment (Figure 90). The nuclear volumes vary periodically in a pattern consistent with the cycling of the mitotic index;⁽¹⁾ gravity compensation introduces a shift in phase between 6 and 12 hr in this periodicity of nuclear volumes.

As with the mitotic indices, we cannot conclude whether the phase shift was caused by the gravity

MEAN NUCLEAR VOLUMES OF X-IRRADIATED
ROOT TIPS OF *VICIA FABA*

FIG. 90—Mean nuclear volumes of interphase cells in the root tips of X-irradiated *Vicia faba* seedlings. Volumes calculated as: S, Spheres; OS, oblate spheroids; PS, prolate spheroids.

FIG. 91—Incidence of micronuclei in interphase cells of the root of *Vicia faba* seedlings after X irradiation. C, Compensated; VR, rotated controls.

treatment or by an interaction between gravity and X radiation. It may be pointed out, however, that at zero time, in samplings made immediately before irradiation, the nuclear volumes of the vertically rotated seedlings were high and progressively decreased. On the other hand, the nuclear volumes of the compensated roots were initially low and progressively increased. Thus it seems likely that shifts in periodicity of both nuclear volume and mitotic index would occur as a result of compensation without X irradiation. Whether compensation itself does have this effect is being investigated.

The occurrence of micronuclei is also influenced by gravitational treatment. Figure 91 shows the incidence of micronuclei in interphase cells at various times after irradiation. There is a significant interaction ($p < 0.01$) between the treatments and time. Between 12 and 30 hr after irradiation, the frequency of micronuclei was significantly higher in those organs that were compensated compared to those vertically rotated ($p < 0.01$). We would expect an increase in the incidence of micronuclei in dividing cells as they pass through their first post-irradiation mitosis.⁽⁵⁾ This was observed. The mitotic index in the compensated roots rose to a peak at 18 hr after irradiation;⁽¹⁾ frequency of micronuclei also increased in this period (Figure 91), reaching a peak at 24 hr, and then decreasing as the cells continue to multiply. Though the variations in number of micronuclei in the vertically rotated roots are not large, their incidence tends to peak at 6 and at 30 hr, times at which the mitotic frequency is high in these organs.⁽¹⁾

Why the number of micronuclei is materially greater

in the compensated roots is not clear. We might assume that the micronuclei in the irradiated tissues were largely derived from one or more lagging acentric fragments; presumably they could also arise from abnormal spindle behavior or from the lagging of centric chromosomes.⁽⁶⁾ Thus one would expect a higher incidence in frequency of aberrations in the compensated tissues some time before the incidence of micronuclei increased, i.e., in the period between irradiation to 12 hr later. That such occurs is not evident (cf. Fig. 180 in Ref. 1); though chromatid and isochromatid breaks, exchanges, total breaks, total aberrations, and aberrant cell frequency at 12 hr after irradiation were all slightly greater in the compensated tissues as compared to those vertically rotated, the incidence was significantly lower in those compensated organs sampled at 6 and 8 hr after irradiation.

It could be that the number of micronuclei was associated specifically with those aberrancies occurring about 12 hr after irradiation, or with anaphase abnormalities undetected at antecedent metaphase. A third interpretation, possibly associated with the preceding, is that the rise in micronuclei with compensation is derived from events unrelated either to irradiation, or to radiation-induced chromosomal damage. It would be of particular interest, therefore, to determine the incidence of micronuclei at various times in unirradiated organs that have been compensated throughout their ontogeny. Though there was no significant difference ascribable to gravity in the amount of Feulgen dye bound per nucleus in the 12- to 24-hr period, the greater number of micronuclei in these same compensated cells suggests the possibility that the total DNA

per cell may have been enhanced by gravity compensation.

CONCLUSIONS

Ignoring single temporal differences the DNA content (as indexed by microspectrophotometry of bound Feulgen dye) of interphase nuclei in the roots of irradiated *Vicia faba* seedlings was not materially affected by gravity compensation. However, gravity compensation appears to shift the circadian periodicities of nuclear volume as well as of mitotic frequency in these cells. Compensation also enhances the incidence of micronuclei in the period between 12 and 30 hr after irradiation.

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RECIPROCITY IN THE GEOTROPIC RESPONSE OF GRAVITY-COMPENSATED AVENA COLEOPTILES

Jane Shen-Miller and Lourdes Morris

PURPOSE AND METHODS

Geotropism in plants grown in normal orientation to gravity follows the reciprocity rule. In other words, for a threshold geotropic response, the product of the stimulation force and time is a constant. Reciprocity has been observed to hold when seedlings are stimulated by various centrifugal forces either with⁽¹⁾ or without⁽²⁾ simultaneous nullification of the directional effect of gravity (compensation). The constant found by Rutten-Pekelharing⁽²⁾ for the shoot was ca. 300 gravity-seconds (*gs*). The experiments of Johnsson⁽¹⁾ yielded a constant of 240 *gs*.

With oat seedlings grown under continuous compensation, we have observed that threshold georesponse of the shoot occurs at ca. 340 *gs*.⁽³⁾ We suggested that this value could be unduly high, since it is not known when geosensing commences in a germinating seedling. The present study was, therefore, undertaken to determine first, whether reciprocity in geotropism holds for plants grown under continuous gravity compensation; second, if reciprocity does hold for these plants how does the threshold magnitude for initial georesponse compare to that of the normal plant; third, the time when geosensing begins in the ontogeny of a compensated seedling.

Oat seedlings are grown in the dark for 48 hr on a 1π horizontal clinostat,⁽⁴⁾ whose rotation (2 rpm) compensates for the directional component of earth gravity. The control seedlings are grown in a normal orientation on a vertical clinostat (2 rpm). The shoots were geotropically stimulated with a 2π clinostat⁽⁴⁾ in order not to interrupt the compensation. Various combinations of stimulation force and time are tested. For each test, the force is held constant and stimulation time varied. Geotropic response of the coleoptile is measured at several intervals after geostimulation and during development of geotropic curvature.

PROGRESS REPORT

Preliminary experiments were run on the time-course of curvature development in geostimulated coleoptiles using various combinations of force and time. The force-time product was kept at a constant 300 gs. The

results are shown in Figure 92. Since appreciable curvatures were noted in both the compensated and vertically-rotated seedlings, the georesponse threshold in these plants appears to be less than 300 gs. Compensation enhanced the curvature response to acceleration. With all treatments, maximum curvatures occurred at 30 or 60 min after stimulation.

An oscillation occurs in the response curves (Figure 92). Interestingly, the oscillation of all treatments appears to be in unison, with a periodicity of about 15 min. Apparently centrifugal stimulation can synchronize the geo-oscillation of the coleoptile. Possibly these oscillations are related to the similar periodicities that have been observed for rates of auxin transport in the normal seedling.⁽⁵⁾

At 1 g, the geotropic response of the compensated shoot is a linear function of the log of stimulation time (Figure 93). This relationship has also been observed

FIG. 92.—Geotropic curvature of *Avena* coleoptiles measured at intervals after stimulation with various combinations of acceleration and time. The acceleration-time product is a constant 300 gs. A, Compensation before and after geostimulation; B, seedlings vertically rotated before and compensated after geostimulation. Acceleration expressed as relative centrifugal force, *g*. Each datum is the mean of at least 30 replicates.

FIG. 93.—Dose-response curve of compensated *Avena* coleoptiles stimulated at 1 *g*. Curvatures were measured at 60 min from start of geostimulation. Each datum is the mean of at least 15 replicates.

for seedlings not continuously compensated.⁽¹⁾ The data in Figure 93 extrapolate to yield a geothreshold of ca. 90 *gs*. This value is approximately $\frac{1}{3}$ of that found for plants not compensated throughout their ontogeny.^(1, 2)

CONCLUSION

The threshold for geotropic response for the shoot of compensated *Avena* seedlings appears to be about $\frac{1}{3}$ less than that of seedlings grown under normal orientation to gravity. An oscillation in georesponse, with a periodicity of 15 min, has been observed. The oscillation appears to be phased by geostimulation or by the combination of geostimulation and subsequent compensation.

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THE CAUSAL BASIS OF GRAVITY STIMULUS NULLIFICATION BY CLINOSTAT ROTATION*

Richard R. Dedolph, Donald A. Oemick, Bobby R. Wilson, and Gilbert R. Smith

Using appropriate clinostat rotation methods, it has been shown that increases in root growth and geotropic curvature of oat coleoptiles are related to and explained (within the limits of auxin economy) by increased rates of plant respiration imparted by nullification of the gravitational stimulus. Increased respiration with decreased gravitational stimulus, in turn, is explained

by increased uniformity in intracellular distribution of metabolically active graviprecipitable particles.

Oat seedlings grown with nullification of the gravitational stimulus show a more uniform distribution of graviprecipitable protoplasmic inclusions. Respiration involves reactions between particles of sufficient mass to be precipitated in $1 \times g$ unidirectional force field and particles of such size that they are distributed throughout the protoplasm owing to thermal energy.

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When plants are grown without nullification of the gravitational stimulus, graviprecipitable protoplasmic inclusions tend to accumulate in the lower portions of the cells. Respiration rates are consequently limited because of a relatively low concentration of the smaller particles in the region of high concentration of the larger particles. Distribution of the larger particles throughout the protoplasm negates the reaction-rate-limiting effects of these intracellular concentration gradients.

It has been shown, using starch particles to index graviprecipitation of intracellular particles, that enhancement of respiration by nullification of the gravitational stimulus may be induced, maintained, discontinued, and reinduced by treatments which con-

comitantly result in the more uniform distribution of graviprecipitable protoplasmic inclusions, the maintenance of this uniform distribution, the discontinuation of this uniform distribution, and its reinduction.

The degree of uniformity of intracellular particle distribution in response to treatments only partially nullifying the gravitational stimulus is shown wholly consonant with growth responses of roots and coleoptiles when the growth is measured as the expression of increased respiration in the absence of auxin limitation.

The findings collectively indicate that gravity sensing by plants is broadly based on the physicochemical relationship between particle distribution and the expression of respiratory metabolism as growth.

THE EFFECT OF SIMULATED LOW-GRAVITY ENVIRONMENTS ON PHOTOSYNTHESIS

*Richard R. Dedolph and Ronald C. Waldbillig**

PURPOSE AND METHODS

Data at hand suggested that increased seedling respiration and dependent growth phenomena are causally linked with increased spatial symmetry in the distribution of graviprecipitable protoplasmic inclusions. Photosynthesis, like respiration, involves reactions between protoplasmic inclusions which are precipitated in the $1 \times g$ force field and particles which, because of

their small size, are distributed because of thermal energy. It, therefore, appeared that continuous reorientation of plants in the gravity force field, resulting in more uniform spatial distribution of the graviprecipitable inclusions and concomitant minimization of reaction-rate limitation from intracellular concentration gradients, might result in increased photosynthesis. To test this, sand-grown 7-day oat plants were attached to lighted horizontal or vertical axis clinostats rotating at 2 rpm (Figure 94). Plants were rotated in darkness

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FIG. 94.—Clinostat with chambers attached and equipped with lights for photosynthesis studies

TABLE 26. THE INFLUENCE OF GRAVITY STIMULUS NULLIFICATION ON CO₂ UPTAKE AND EVOLUTION BY 8-DAY-OLD OAT SEEDLINGS^(a)

	Unidirectional gravity stimulus, g	
	0	1
Uptake (light)	901.9	830.3
Evolution (dark)	452.7	413.6
Net gain in fixed carbon	449.2	416.7

^(a) Data are mg CO₂/kg fresh weight. All means within rows are significantly different at the 1% level.

for about 15 hr, then subjected to 4 sequential 3-hr regimes of 1.5 hr of darkness, followed by 1.5 hr of light. Twice during each light and dark period in each regime, the CO₂ content of the metered transient air stream was measured with a CO₂ gas analyzer. Temperatures surrounding the clinostats and of the influent and effluent air streams were maintained at 25° ± 1°.

THE EFFECT OF GRAVITY COMPENSATION ON THE SILKWORM (*BOMBYX MORI*)

Jane Shen-Miller and Patricia Cooper

PURPOSE AND METHODS

Little is known of the effect of continuous reorientation to gravity on invertebrate development. This study was undertaken to examine the effect of such motion on the metamorphosis of the pupa of the silkworm (*Bombyx mori*). Larvae were hatched 12 days after eggs were removed from cold storage (4–6°C). The worms were fed fresh mulberry leaves for ca. 30 days. During this period they underwent 4 moults and then pupated (cf. Ref. 1). Immediately upon completion of the cocoon it was fastened with Scotch tape to the center of the bottom of a 250-ml beaker, which was then placed on a 1 π clinostat⁽²⁾ for the gravity treatment. The beakers were rotated either on a horizontal (gravity compensation, C), or vertical axis of rotation (control, R), or allowed to remain in a vertical stationary position (S). The rotation rate was 1 rpm. As the moth emerged, it was weighed and its appearance examined. One male and one female were placed in a beaker lined with tissue paper to provide footing. Rotation was continued during syngamy and egg-laying. Approximately 10 days after mating the eggs were collected.

PROGRESS REPORT

In the initial experiment (Table 27, Expt. I), an increased percentage of abnormal moths was noted in the

Respiration, photosynthesis, and net carbon fixation by plants removed from unidirectional gravity stimulus were materially increased as compared to plants subjected to this stress (Table 26). Increased respiration, photosynthesis, and net carbon fixation due to removal of plants from the unidirectional gravitational stimulus were not affected by time of measurement (i.e., the increases were consistently maintained from the first through the last of the 4 sequential regimes). This consistency of response over time suggests that the size of the metabolic system of these 8-day old plants was not materially altered during this short (12-hr) experimental period.

CONCLUSION

It is apparent from these data that photosynthesis increases attributable to nullification of the unidirectional gravity stimulus are consonant with respiration increases and likely share a common source and causal basis.

C treatment. It was uncertain whether these abnormalities resulted from rotation per se or from mechanical injury in tumbling of the pupae in the cocoons. Accordingly, in a second experiment, soon after a cocoon was spun, one-half of it was cut open along its longitudinal axis. At this time the larva had just finished spinning. The quiescent worm was weighed and returned to its silk shell into which cotton had been loosely padded. (The padding prevented tumbling of the pupa during rotation.) The cocoon was then taped shut and attached to a beaker for treatment as in Experiment I. Table 27 shows the results of the two experiments.

Padding of the cocoon reduced the incidence of abnormal adults in the compensated treatment (Expt. II, compare C and R). There is no significant difference in the times required for the development of pupa to moth and in the number of moths emerging among the treatments. In both experiments the life-spans of the compensated moths were consistently shorter than those of the moths from R and S treatments, particularly in the females. However, these differences do not appear to be significant statistically in the population numbers tested. Egg production of the female moth was not affected.

TABLE 27. THE EFFECT OF GRAVITY COMPENSATION ON THE SILK WORM

	I ^(a)			II ^(a)			Mean		
	C	R	S	C	R	S	C	R	S
♀									
Adult emergence, days	16.5	17.1	17.5	16.0	15.8	16.4	16.2	16.4	17.0
“ “ %	83.3	87.5	87.5	100	96.7	96.7	91.6	92.1	92.1
Larval weight, ^(b) g	2.58	2.62	2.50	1.66	1.63	1.59	2.12	2.12	2.04
Adult weight, g	0.83	0.92	0.74	0.96	0.85	0.84	0.90	0.88	0.79
Adult's life-span, ^(c) days	4.4 ± 1.4	8.4 ± 2.1	8.3 ± 1.9	8.8 ± 1.0	10.8 ± 1.2	12.2 ± 1.0	6.6	9.6	10.2
Eggs per moth	367	459	509	558	510	522	462	484	516
Abnormal moths, %	70.0	28.6	16.7	46.2	42.8	27.3	58.1	35.7	22.0
♂									
Adult emergence, days	17.5	15.9	16.5	15.7	16.0	15.9	16.6	16.0	16.2
“ “ %	83.3	87.5	87.5	100	96.7	96.7	91.6	92.1	92.1
Larval weight, ^(b) g	2.34	2.37	2.35	1.30	1.35	1.35	1.82	1.86	1.85
Adult weight, g	0.46	0.42	0.40	0.45	0.48	0.44	0.46	0.45	0.42
Adult's life-span, ^(c) days	14.1 ± 1.1	15.0 ± 1.0	15.9 ± 0.6	12.6 ± 0.7	13.8 ± 0.6	14.0 ± 0.8	13.4	14.4	15.0
Abnormal moths, %	10.0	14.3	0	29.4	27.3	5.9	19.7	20.8	3.0

^(a) Exp. I: 24 cocoons per treatment; Exp. II: 30 cocoons per treatment.

^(b) Exp. I: Before spinning cocoon; Exp. II: After spinning cocoon.

^(c) ± Standard error of the mean.

CONCLUSION

Silkworms were maintained on clinostats from the time of pupation to the mating and egg-laying of the adults. Continuous reorientation with respect to the direction of the gravitational field had no material effect on the time required for pupation and the percentage of adult emergence. The life-spans of the adult were shortened by gravity compensation; further work

is required to establish the statistical validity of this inference.

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Mammalian Genetics

MAMMALIAN GENETICS: GENETICS OF VIABILITY IN THE MOUSE

I. REPRODUCTION PERFORMANCE

Douglas Grahn, Katherine F. Hamilton, and Wayne P. Leslie

PURPOSE AND METHODS

There are two principal motivations for the study of the genetics of viability. One, the past several decades of experience in the analysis of radiation injury in mice has indicated the need to integrate all genetic aspects of viability, in all periods of the life cycle, into the interpretation of short-term and long-term radiation injury and recovery. Life expectancy, instantaneous rates of mortality, causes of death, tissue and cellular recovery are all under some degree of genetic control and, therefore, the genotype influences all interpretations of radiation damage parameters. The second motivation follows from the first: there is simply a great deficiency of knowledge concerning the basic genetics of viability. A recent review⁽¹⁾ substantiates the many biological mechanisms through which genetic influence on ageing may be exerted, but also clearly indicates that no genetic understanding is apparent.

This study will draw upon the accumulation of lifetime records of diverse inbred mouse strains, genetic marker stocks, hybrids, and backcross generations among selected inbreds. The data include survival and pathology statistics on many strains and hybrids following single dose whole-body X and gamma irradiation, duration-of-life daily gamma irradiation and unirradiated control populations. The first task will be to reduce and analyze the existing data for both direct and serendipitous information; all data were not specifically collected for the purposes in mind. Subsequently, direct examination of established hypotheses can be carried out.

Our data collection falls into three periods of the lifetime, and the analysis will have to follow this preset pattern. The periods are: birth to weaning (about 30 days of age); weaning to 100 days of age; 100 days to death. The reason for this pattern is that most of our radiation studies began with 100-day old mice and the period from 30 to 100 days was used to accumulate

mortality experience during a time of normally low death rate.

The first effort logically concerns reproduction. Mating records spanning a 15-yr period and including 20,000 litters are available. These are at present being subjected to a first analysis to define the major variables and the genetically significant correlations that may exist.

PROGRESS REPORT

We are studying the following variables of the reproductive performance (initially only the data on the BALB/c, C57BL/6, and (C57BL: BALB/c)F₁ are being examined to determine the major interrelations).

Dependent variables:

Neonatal and infant mortality (preweaning)

Litter interval

Sex ratio

Independent variables:

Parity

Maternal age

Calendar month

Calendar year

Dependent and independent:

Litter size.

All data are available by sex, strain, hybrid or other genetic class. Early mortality can be expressed as total death rate or as the rate by age intervals: 0-5, 6-15, 16-30 days.

Figures 95-100 present some of the major relationships. Data for the two sexes are combined as there is surprisingly little difference between them in early mortality. Figure 95 demonstrates a standard mammalian early death rate curve, neonatal mortality (0-5 days) being the highest. An unexpected observation is that relating early death to maternal age. Experience in man shows a parabolic curve with a definite minimum at 25 to 30 yrs and a rising death rate among offspring from younger or older mothers. The

steadily rising death rate seen in Figure 97, from a minimum at youngest maternal ages, clearly suggests a different maturation-aging pattern for the mouse.

Another somewhat unexpected finding is the high death rate in all small litters—those below the mean litter size. The relation between litter size and maternal age (Figure 96) is well known and in part explains the

FIG. 95—(Upper) Average daily death rate versus cohort age for all litters.

FIG. 96—(Lower) Maternal age litter size relationship

FIG. 97—(Left) Average daily death rate over whole preweaning period (0-30 days) for all litters versus maternal age.

FIG. 98—(Right) Average daily death rate (as in Figure 97) versus litter size at birth.

FIG. 99—(Upper) Mean litter size at birth for all litters versus calendar year.

FIG. 100—(Lower) Average daily death rate over whole preweaning period (0-30 days) versus calendar year.

litter size-mortality relation. A multivariate analysis of these three variables is required to isolate maternal age effects on offspring mortality.

The relationship of mortality and litter size with calendar year demonstrates the stability of both the laboratory environment and the genetically differentiated strains. Hybrid vigor is noted significantly in only one parameter—litter size, synonymous with yield or productivity in economic plants and animals. One additional small hybrid advantage exists in the lower neonatal mortality rate for F_1 progeny from dams less than 250 days of age. Otherwise, the BCF_1 is very similar to the $BALB/c$ parent, yet this parent is the male parent of the outcross. This similarity to the male parent is also noted for sex ratio.

CONCLUSIONS

A study of the genetics of viability has been initiated with first observations on attributes of reproductive performance. Significant correlations exist between early mortality and maternal age, litter size, and cohort age. Litter size also varies nonlinearly with maternal age. Hybrid vigor is only strikingly seen in litter size. A sharp contrast also exists between man and mouse regarding maternal aging effects upon offspring survival.

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MAMMALIAN GENETICS: DETERMINATION OF THE RADIATION-INDUCED MUTATION RATE FOR SEX-LINKED RECESSIVE LETHALS AND VISIBLE AUTOSOMAL GENES IN THE MOUSE

Douglas Grahn, Rebecca Craggs, and Wayne P. Leslie

PURPOSE AND METHODS

Previous reports^(1, 2) have described the experimental methods employed to ascertain the radiation induced mutation rate for sex-linked genes in the mouse. Some modifications of the experimental details are planned and discussed in the next section, but these do not change the basic pattern of the test system presented in these earlier reports.

PROGRESS REPORT

The cumulative results to date of the F_1 and F_2 generations are given in Table 28. A total of 327 X-chromosomes have been screened through the two generations, an increase of 183 during the past year. The incidence of suspects continues to be higher for the irradiated chromosomes than for the controls, 6.0% vs. 3.4%.

There have been 15 suspects identified, but only 12 have provided sufficient female F_2 offspring for further test. Tests on 9 of these suspects are incomplete; 2 suspects were discarded as false positives by virtue of the lack of sex-ratio deviation among F_3 progeny, and one was discarded as negative in the F_4 linkage test. Thus, positive genetic evidence for the occurrence of a lethal mutation has yet to be secured.

Ten F_2 daughters are usually tested in a suspect family to assure a 0.999 chance of recovery of at least one F_2 carrier. Curiously, of 7 suspect families that have completed most of the screening through the F_3 generation, only 6 F_2 daughters among 67 tested, or 9%, have produced F_3 families with a sufficient deficiency of males to warrant their classification as carriers. We are, therefore, identifying an excessive number of false positives. The reasons are partly understandable—our probability statements are calculated from an expected ratio of 1.7:1 (σ : δ) as the standard based upon early experience, but the observed ratio is at present closer to 2:1. Thus, more

families reach the truncation line of 1.4% false positives (for families of 30 F_2 offspring) and are subject to further test.

The observed frequency of suspects in the controls, 3.4%, is almost exactly the expected frequency, 3.5%, for families of 30 that would meet the criterion of having 5 males or fewer, when the p values are calculated for a basic 2:1 sex-ratio. The greater frequency among the irradiated families, 6%, may, therefore, include induced detrimental genetic damage. The excess over controls, $\sim 2.5\%$ or 0.025 per 500 rads, suggests an induced rate for lethals of 5×10^{-5} /rad/X-chromosome. We must reemphasize, however, the observation that we have not been able to isolate genetically any presumed mutations.

The original probability conditions are being maintained, however, since this element of conservatism reduces the probability of discarding false negatives during this early period of the study. Some modifications of the test system will make the screening of the F_2 , F_3 , and later generations more efficient, while providing a nearly absolute screening of a small portion of the X-chromosome for mutation.

To date, there have been no detected mutations at the autosomal loci. We will continue to screen for mutation at several loci in the F_1 , but the F_2 screening will gradually drop out as changes in the overall screening procedure are made.

As data on the F_1 and F_2 generations accumulate, analyses of additional attributes of reproductive performance become feasible. There is, for example, a slightly higher rate of sterility or poor fecundity due to early death or failure to have successive parities among the F_1 dams carrying the irradiated genome. The present figures are 14% versus 10%. It has not yet been possible to make significant comparisons of the survival of F_1 and F_2 progeny between birth and weaning for the irradiated and control groups. As data

TABLE 28. SUMMARY OF DATA

	Autosomal genes											Number of sex chromosomes		
	$n^{(a)}$	<i>A</i>	<i>n</i>	<i>b</i>	<i>c</i>	<i>n</i>	<i>a^e</i>	<i>bl</i>	<i>ln</i>	<i>p</i>	<i>wa-2</i>	Tested	Suspect	% Suspect
Irradiated	1002	—	639	—	—	248	—	—	—	—	—	149	9	6.0
Control	1733	—	1406	—	—	318	—	—	—	—	—	178	6	3.4

^(a) Number of gametes tested.

continue to accrue these and other measures of induced genetic damage will be made.

Proposed Modifications in the Mating and Screening System

Although the method now employed has continued to give evidence for the presence of induced sex-linked lethals, the difficulty in their detection is discouraging. Two major factors are possibly acting to reduce the sensitivity of the system—the use of an unmarked irradiated X-chromosome and a potential viability effect upon the carrier female. To overcome these deficiencies partly, we are planning to use the sex-linked gene “greasy” (*Gs*) to mark the irradiated X-chromosome. This gene is unusual in that it has no detectable effect on viability. The irradiated *Gs* male will then be mated to a brindled (*Mo^{br}*) female, instead of the tortoise gene as now done. *Mo^{br}* has less effect on viability of the female and permits the carrier males to survive through birth and up to about two weeks of age. This reduces the potential burden of prenatal death upon the dam who may be carrying in addition a new lethal. Thus, the F_1 dam that will be screened as a carrier will have the genotype $+Mo^{br}/Gs+$. A new lethal will not only affect sex ratio but it will also be accompanied by a deficiency of *Gs* males. The short region between *Gs* and *Mo^{br}*, about 4% recombination, will be virtually under absolute screening—no F_2 males should

survive to weaning except those resulting from a double crossover (4 in 10,000). These changes should be invoked during the coming year.

CONCLUSIONS

The study to determine the radiation induced mutation rate for sex-linked lethals has screened 327 chromosomes. Six percent of the irradiated chromosomes and 3.4% of the controls have been identified as suspect carriers of new lethals. The control figure is equal to that for the random expectation of false positives which suggests the excess among the irradiated families may be associated with detrimental mutations. We have not yet been able to isolate and genetically identify a new lethal mutant, however. Several changes in the mating system are being instituted to increase the efficiency of the test.

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MAMMALIAN GENETICS: AN UNUSUAL OCCURRENCE OF XXY TORTOISE MALES AND PRESUMED XY//XY MOSAIC

Douglas Grahn and Rebecca Craggs

In the study to determine the radiation induced mutation rate for sex-linked lethals,⁽¹⁾ four instances of males carrying the sex-linked lethal gene “tortoise” have occurred. Although the occurrence of XXY males, which could explain these events, is not unknown, their appearance within a brief four-month period and thus with an apparently high short-term frequency makes this an unusual event.

The four tortoise males were derived from the following typical genetic history:

P	$\frac{T_o}{+}$	×	$\frac{+}{+}$	(Irradiated or control)
		↓		
$\downarrow$	$\frac{T_o}{+}$	×	$\frac{+}{+}$	(Marker stock carrying six recessive autosomal genes: <i>a^o</i> , <i>b</i> , <i>bt</i> , <i>ln</i> , <i>p</i> , <i>wa-2</i> . Strain designated as 7-pl)
		↓		
F_2	<i>T_o</i> male			

Table 29 gives the pertinent details of the four events. It is evident that the only common factor is the marker stock, 7-pl, as the male parent and, of course, the *T_o* gene in the F_1 dam. Unfortunately, only one of the three X-chromosomes in the crosses is marked.

Tortoise male No. 1 was placed in mating in mid-June and proved to be fertile. A number of different dams have been employed, and a summary of the data is in Table 30. A nearly perfect sex ratio is noted, while the segregation ratio for the *T_o* vs. the unmarked X is about 3:1. This male could be an XY//XY mosaic with about a 3:1 ratio of *T_o*/Y//+/Y in the germinal tissue. No apparently unusual offspring have been detected among the progeny from the *T_a*/*T_a* or *T_a*/+ dams. Thus, the two X-chromosomes in the tortoise male appear to segregate quite separately and without any suggestion of aberration.

TABLE 29. ATTRIBUTES ASSOCIATED WITH OCCURRENCE OF TORTOISE MALES

Attributes	Tortoise ♂ number			
	1	2	3	4
Grand sire	C57BL/6	C57BL/6	C57BL/6	A/Jax
Grand dam	A/ <i>To</i>	AF/ <i>To</i> ^(b)	A/ <i>To</i>	C57BL/ <i>To</i>
Radiation dose to grandsire	500 R	500 R	0 R	500 R
Sire	7-pl ^(a)	7-pl ^(a)	7-pl ^(a)	7-pl ^(a)
Dam	ABF ₁ <i>To</i> /+	AFBF ₁ <i>To</i> /+	ABF ₁ <i>To</i> /+	BAF ₁ <i>To</i> /+
Age of sire	326 days	190 days	251 days	97 days
Age of dam	190 days	172 days	193 days	315 days
Parity	1	3	2	6
Date born	4-9-67	4-23-67	6-20-67	8-11-67
Phenotype	Non-ag. brown tortoise	Non-ag. black tortoise	Non-ag. brown tortoise	Non-ag. brown tortoise
Breeding history	Fertile	Sterile	Prob. Sterile	—
Total F ₂ offspring				
Observed in type cross	047	826	1822	1250

^(a) Genetic marker stock carrying 6 recessive autosomal genes: *a*^o, *b*, *bl*, *ln*, *p*, *wa*-2.

^(b) Tortoise stock maintained by recurrently crossing the *To* gene to the A/Jax and the CF #1/Anl strains in a criss-cross manner

TABLE 30. Offspring of *To* ♂ No. 1

Dam's genotype	No. parities	No. of offspring		Classification													
				♂					♀								
		♂	♀	Und. ^(a)	<i>Ta</i>	+	<i>Gs</i>	<i>Mo</i> ^{br}	Und.	<i>To</i> / <i>Ta</i>	<i>To</i> /+	<i>Ta</i> /+	+/+	<i>To</i> / <i>Gs</i>	<i>To</i> / <i>Mo</i> ^{br}	<i>Gs</i> /+	<i>Mo</i> ^{br} /+
<i>Ta</i> / <i>Ta</i>	7	26	19	2	24	—	—	—	1	12	—	6	—	—	—	—	—
<i>Ta</i> /+	2	6	13	1	1	4	—	—	0	6	3	0	4	—	—	—	—
<i>c/c</i> +/+	2	7	8	0	—	7	—	—	1	—	6	—	1	—	—	—	—
+ <i>Mo</i> ^{br} / <i>Gs</i> +	1	5	2	0	—	0	3	2	0	—	—	—	—	1	1	0	0
Total	12	44	42														

^(a) Undetermined.

The mating with *Mo*^{br} is specifically a test for allelism. The *To* gene in this laboratory occurred spontaneously in 1958 in the C57BL/6 stock and has only been genetically tested by its linkage relation to *Ta*. That test indicated a 4% recombination; the present test is unequivocal. One pale gray female, which appeared phenotypically identical to the *Mo*^{br} male, occurred in the first litter. Death occurred at about 10 days but the interpretation is that she was a *Mo*^{br}/*To* female. Since it is also conceivable that the animal could have been an XO (*Mo*^{br}/O) additional matings will be made with *Mo*^{br}, *Mo*^{dp}, *Blo*, and *To*, itself. Since our present data on time of death of *To* males indicates this occurs post-implantation and prenatally, it is not expected that *To*/*To* females would be detected or that even *Mo*^{dp}/*To* females would survive to birth. Nevertheless, this opportunity to make direct tests of allelism at this phenotypically if not genetically, complicated locus cannot be passed by.

The matings with the albino dams are to test the possibility that the albino gene is carried in the portion of the germinal tissues that may be +/Y. Since this X-chromosome could theoretically have been of either maternal or paternal origin, this doubt could be eliminated if a non-tortoise albino female is produced. The logic behind this is based on the autosomal markers involved in the genetic history of this *To* male. This history is as follows (since the *To* ♂ is *a/a b/b* or non-agouti brown, not all possible gametes are shown for the F₁):

P *a/a b/b c/c To*/+ × *a/a B/B C/C* +/Y

F₁ *a/a B/b C/c To*/+^m × *a^o/a^o b/b C/C* +^p/Y.

F₁ gametes: *a b^c C To* *a^o b C +^p*
 a b C +^m *a^o b C Y*
 a b c To *a^o b C +^pY* (non-disjunction
 a b c +^m gamete)

If this *To* male is an XY//XY mosaic derived by the

separate fertilization by Y-bearing sperm of both second meiotic division products, there is one chance in two that the unmarked X is associated with the albino gene (the fourth possible ♀ gamete above). The occurrence of an albino non-tortoise female would prove the maternal origin of this X. If an albino *To* female or albino male should occur, or if no albino should appear at all, the test is merely indeterminate.

The possibility of dispermy involving a normal Y-bearing sperm and a normal second division ovum plus a non-disjunction XY-bearing sperm seems very remote, if not denied by the fact that the whole mouse is apparently a mottled brown and tortoise. If a male non-disjunction gamete made up the non-*To* portion of the mosaic, the autosomes would be haploid, and portions of the animal should show leaden and wavy hair. The pink eye dilution and the belt spotting traits might be less easy to discern in the mottled tortoise animal. Since the mouse does show some diffuse pale areas that might be interpreted as leaden-brown, a backcross to the 7-pl marker stock is being made. If the unmarked X segregates with all six autosomal traits of the 7-pl always expressed, then that portion of the mouse would be an autosomal haploid (or diploid) plus X^pY^p with the genome entirely derived from 7-pl. The occurrence of any non-tortoise female failing to express any or all of the traits (belt, leaden, pink eye and waved on the non-agouti, brown base) immediately denies the interpretation that one portion of the mosaic is entirely derived from a non-disjunction male gamete.

The male tortoise No. 2 was mated without success to 6 or 8 different females over a three-month period. He was sacrificed, and a number of tissues were prepared for karyotype analysis by Dr. R. J. M. Fry of this laboratory, and successful cell cultures were also obtained by Dr. A. N. Stroud. Corneal tissue metaphase plates yielded, in 50 separate counts, a 50% frequency of 41 chromosome configurations, 24% 40 chromosomes, and the balance scattered at lower and higher numbers. The gonads contained apparently functional interstitial tissue, but the tubules contained only Sertoli nurse cells, no germinal cells. The mouse is presumed to have been an XXY.

Male tortoise No. 3 at present in mating and appears to be sterile since no progeny have been produced in over a six-week period. He will also be subject to cytological analysis.

Male No. 4 has not yet been put into mating.

CONCLUSIONS

Tortoise male mouse No. 2 definitely has 41 chromosomes and is undoubtedly an XXY male. Male No. 3

is probably the same, since it is apparently sterile. Male No. 1 presents the problem of interpretation. The subject of abnormal fertilization events, genetic mosaics, polyploidy in mammals, etc. has been studied and reviewed extensively.^(2, 3, 4) A fertile tortoise male can have one of several possible genetic origins, but he at least must be a mosaic or chimera probably of $1n/2n$ or $2n/2n$ tissue makeup. The segregation of two X-chromosomes and the Y-chromosome with a nearly equal sex ratio among the offspring favors the $2n/2n$ interpretation. The animal may, therefore, be $\overline{XY}/XY$, or more specifically, $\overline{To}/\overline{+}$. The ratio of *To* to unmarked X-chromosomes is about 3:1. There is no way of judging the mosaic ratio phenotypically since the animal has the general appearance of a normal brown tortoise mouse with $50\% \pm 25\%$ of the coat color of one type or the other. The remaining question is then, what is the origin of the unmarked X—maternal or paternal?

The following possibilities must be considered if both X-chromosomes are maternal.

- Immediate cleavage at the first meiotic division followed by fertilization of both ova with Y-bearing sperm, normal polar-body extrusion, and fusion of the two ova into a single zygote.
- Immediate cleavage at the second meiotic division, preceded immediately by dispermy and the formation of two fused but separately fertilized ova.
- An $XX//XY$ mosaic is unlikely since gynadromorphs are sterile. An $XO//XY$ is also unlikely due to the fertility of this male.

The unmarked X could only be of male origin if an XY non-disjunction gamete became involved in the formation of the mosaic. This can be genetically tested by backcrossing to the male parent and examining the progeny for segregation at the marked autosomal loci.

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INHERITED CONNECTIVE TISSUE DEFECT IN TORTOISE MICE

R. J. Michael Fry, Bernard Slaughter, Douglas Grahn, Friedrich Wassermann, Faustina Manelis, Katherine F. Hamilton, and Everett Staffeldt

PURPOSE AND METHODS

Studies on the tortoise gene, which is a sex-linked lethal gene maintained on different genetic backgrounds in this laboratory, have been reported.^(1, 2) In the more recent report we described the gross form and incidence of the vascular abnormalities, namely aneurysms and an abnormal kink in the thoracic aorta. This report is concerned with two aspects of our continuing investigations. First, we wanted to know whether the sons of dams carrying the tortoise gene, which were found dead on the first postpartum day, were, in fact, stillborn and if any vascular abnormality was present. Lungs were tested for post-partum inflation by simply placing them in water and noting whether they floated. Lungs from known live newborns were used as controls. Examination of the cardiovascular system was made with a dissecting microscope and abnormalities noted; tissues were prepared for histological examination. Secondly, we wish to illustrate the histological and electron microscopic appearance of the wall of the aorta in these cases. As the photographs show, the form though not the nature of the lesion is clear, so a minimum of description will be given.

Sections for light and electron microscopic examination were prepared by standard techniques.

PROGRESS REPORT

Table 31 shows the results for the incidence of aortic abnormalities, apparent hemorrhage, and lung aeration in relation to the whisker phenotype. The tortoise gene results in curly vibrissae, as well as coat color

TABLE 31. INCIDENCE OF AORTIC ABNORMALITIES, APPARENT HEMORRHAGE, AND LUNG AERATION IN RELATION TO WHISKER PHENOTYPE

Whisker phenotype	No. of male mice	Aorta		Hemorrhage		Percent pre- sumed stillborn by flotation test	
		Percent incidence of aneu- rysms	Percent incidence of kinks	Percent incidence			
				Tho- racic	Ab- domi- nal		
Apparent <i>To</i>	12	50.0	83.0	25.0	12.0	70.0	7/10
Straight	5	0.0	0.0	0.0	20.0	100.0	3/3
Indefinite	14	36.0	79.0	14.0	14.0	92.0	11/12

FIG. 101.—Examples of the abnormalities seen in male tortoise mice.

changes. In the newborn, vibrissae are often difficult to detect; thus in the majority of the mice the whiskers could not be classified. The important point of these findings is that the vascular abnormalities previously noted only in the female are found in the male at term. The incidence of earlier intrauterine death has been studied, but now it is necessary to establish the precise cause and timing of the death of males. Four phenotypically male mice survived to maturity (but genetically abnormal tortoise) and are discussed in a separate report. Figure 101 shows diagrammatically examples of the aortic abnormalities found in the newborn males. The *kinks* and *dilatations* are more diverse in form than those seen in the females.

FIG. 102.—a, Photomicrograph of 38-day-old normal mouse. Section stained with orcein. Note the sharply defined elastic laminae. b, Photomicrograph of 26-day-old female *To* mouse, orcein stain. Note irregular outline of elastic laminae and the increased interlaminal space. The wall is thicker than normal. c, Photomicrograph of wall of aneurysm of aorta. There is marked fragmentation and splitting of the elastic laminae. There is lymphocytic infiltration of the adventitia.

FIG. 103.—Electron micrographs of thoracic aorta, Epon embedded. a, Tunica media of normal 9-day-old mouse. The elastic lamina can be seen as broad white bands. b, Tunica intima and inner tunica media of 9-day-old tortoise female mouse. Elastic lamina more irregular and widely spaced than normal. c, Tunica adventitia and outer part of tunica media of adult normal mouse. d, Tunica intima and inner tunica media of adult tortoise mouse. Elastic laminae indistinct and fragmented.

The photographs (Figures 102 and 103) show examples of the abnormal structure seen in sections of the aorta. The lesions range from frank rupture of an aneurysm to thickening of the wall with an increase in the interlaminae space. Fragmentation of the elastic laminae was seen in the older animals and in some of the aneurysms (Figure 102c and 103d).

CONCLUSION

Histochemical investigation of both the aortae and fibroblasts has not yet revealed whether the changes in the elastic tissue are primary or secondary to an abnormality of the mucopolysaccharides of the ground substance. In a small number of cases the presence of

metachromasia has supported the possibility of the inherited disorder being related to the mucopolysaccharides.

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ANALYSIS OF FAMILIAL INFLUENCE ON MEAN ERYTHROCYTE VOLUME

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Jeanne A. Pagels, and Merlin H. Dipert*

PURPOSE AND METHODS

Starling⁽¹⁾ described a method for determining the average volume of erythrocytes, a method that Wintrobe⁽²⁾ used so effectively it is universally termed the Wintrobe MCV (mean corpuscular volume). The latter author also provided a distribution curve of MCV values for one hundred healthy young men where the average MCV was $79.8 \mu^3$, and the range was from approximately 60 to $95 \mu^3$. This cell volume distribution curve appears to be bimodal, or even trimodal, and there are no significant correlations for cell volume with race or national origin. Richardson,⁽³⁾ much earlier, had made attempts to identify different races of mankind with variants in cell size, measured by micrometry, but was frustrated by large normal variations within each racial sample. No cell volume frequency distribution curves for normal dogs have appeared in the literature.

In a previous report,⁽⁴⁾ some evidence was presented for a positive correlation between MCV and ancestral relationship. The means of three MCV values for each of 354 purebred beagles were plotted in a frequency distribution curve. The three MCV values for each dog were calculated from hemograms obtained at weekly intervals in the age period of 12 to 13.5 months. This test group of 354 beagles was divided into subpopulations based upon their relationship to the progenitors of the two inbred families within the total population of the colony. In the previous report, these population subdivisions were made upon the basis of parent and grandparent derivation from the

inbred families. In the present report, the data have been subjected to more detailed analysis to determine if MCV is influenced by genes contributed by progenitors of the A and B inbred families.

A program for computing inbreeding coefficients⁽⁵⁾ has been expanded to calculate the percentage contribution of Alabama family (A-family) and Blonde family (B-family) progenitors to the genotypes of each of the 354 dogs in this sample. The contributions of A- or B-relatedness are determined by the relationship proximity of all individuals in six ancestral generations to the A and B progenitors. There are two minor ancestral components in the colony which are not represented in either the A or B inbred families: one of these includes known ancestors other than those in either inbred family, and the other is simply unknown, where pedigrees were incomplete or unavailable for some of the dogs purchased as initial breeding animals.

PROGRESS REPORT

Although the A and B families are being inbred, the lines are not distinct, and many of the 354 dogs of the sample are genetically related to the foundation animals of both inbred families. Construction of frequency histograms for the A-related, B-related, and other dogs suggested that the A-related dogs had a smaller MCV than the B-family, which appeared to be similar to the dogs of unknown lineage. In order to determine whether or not genes derived from the A-progenitors caused a significantly smaller MCV, a variety of regression analyses were performed. The basic data for

TABLE 32. MEAN CELL VOLUMES (MCV) IN RELATION TO A-LINE RELATEDNESS (X_1) AND B-LINE RELATEDNESS (X_2)

X_1	No.	MCV, μ^3	X_2	No.	MCV, μ^3
0.00	133	68.9	0.00	104	64.8
0.25	76	66.1	0.125	54	65.6
0.50	47	65.6	0.187	21	67.7
0.75	23	62.6	0.22-0.25	31	67.9
1.00	75	64.7	0.31-0.34	19	67.1
			0.36-0.38	14	67.4
			0.44-0.48	36	68.5
			0.50	27	67.2
			0.53-0.56	18	67.2
			0.625	18	65.8
			0.94-1.00	12	72.2

FIG. 104.—Graph of observed and expected values of mean cell volume for linear and quadratic regression on A-relatedness.

the present analysis are determinations of MCV (Y), the A-relatedness (X_1), and the B-relatedness (X_2) for the 354 beagle dogs. Table 32 presents the MCV's for various values of X_1 and X_2 .

Simple linear regression coefficients of Y (MCV)

on X_1 (A-relatedness) and X_2 (B-relatedness) were statistically significant and of opposite sign, the coefficient for X_1 being -4.50 ± 1.49 and for X_2 being 5.41 ± 1.37 . These significant regressions clearly indicate a genetic effect on MCV, but since as X_1 increases for a particular dog, X_2 decreases, it is uncertain whether genes derived from the A- or B-progenitors cause smaller or larger erythrocytes, respectively, or whether both effects occur.

Accordingly, a multiple linear regression analysis was performed, yielding the equation $Y = 67.75 \pm 0.56 - (3.93 \pm 0.72)X_1 + (1.36 \pm 1.13)X_2$. Since the regression on X_1 is significant, whereas that on X_2 is not, it is concluded that genes contributed by the A-progenitors cause a smaller MCV, whereas the degree of genetic relationship to the B-family progenitors has no significant effect on MCV.

To examine for possible nonlinear and covariance effects on MCV, an equation of the form

$$Y = a_0 + a_1X_1 + a_2X_2 + a_3X_1X_2 + a_4X_1^2 + a_5X_2^2$$

was fitted to the data. The only significant regression coefficients were $a_1(-15.31 \pm 3.89)$ and $a_4(10.24 \pm 3.32)$. The remaining coefficients were $a_2 = 5.64 \pm 4.01$, $a_3 = 7.81 \pm 7.19$, and $a_5 = 6.95 \pm 3.82$. It appears that only genes derived from the A-progenitors influence MCV significantly, there being no evidence for a significant effect of B-relatedness nor of any interaction between A-derived and B-derived genes.

Accordingly, a second-degree regression equation on X_1 was calculated, yielding $Y = 68.96 \pm 0.33 - (12.59 \pm 2.01)X_1 + (8.27 \pm 1.99)X_1^2$. The linear and quadratic regression coefficients are both significant, clearly indicating that the genes contributed by the A-progenitors do affect the MCV. The significant quadratic term suggests that gene interactions more complex than simple additive effects are also operative. Figure 104 shows the observed and expected values of MCV for both the linear and quadratic regression equations.

Despite the fact that there is a definite genetic component in determination of MCV, most of the variation in MCV is of random origin since only 20% of the total variance can be ascribed to quadratic regression on A-relatedness.

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THE DEVELOPMENT OF AN INBRED ACATALASEMIC MOUSE

Robert J. Flynn, Ronald W. Camden, Robert N. Feinstein, and Douglas Grahm

PURPOSE AND METHODS

The development of an acatalasemic strain of mice has provided a research tool to test certain theories of metabolism, radiation effects, and oncology.^(1, 2) This strain was developed by selecting and breeding solely for the one characteristic, blood catalase level. It was not inbred for any other characteristic. Consequently, the animals exhibit poor reproductivity, lack uniformity of size, and show great variations in coat color.

Inbred strains of mice are available that are uniform in external appearance and size. We are now introducing the acatalasemic locus (Cs^b) into one such strain, C3Hf/Anl[Anl 66], which we are crossing with agouti-colored acatalasemic mice (Cn-1/Anl). The progeny of this initial cross are all hypocatalasemic (heterozygotes for the Cs^b locus). These will be backcrossed to the C3Hf for at least eight more generations. Each backcross generation will be tested (for blood catalase level), and only the hypocatalasemic animals will be used for the next generation. The resultant hypocatalasemic C3Hf nucleus (after eight generations of backcrossing) will be sib mated, and the acatalasemic progeny (homozygous for the Cs^b locus) will be maintained by sib matings. While developing the nucleus of this strain, productivity will be stressed.

For this reason, an attempt will be made to select animals for further breeding only from litters in which nine or more are born and weaned.

PROGRESS REPORT

Ten agouti, CN-1 males have been progeny tested by mating with marker strain females. Three were found desirable for further breeding in that they produced no non-agouti offspring. These were mated with 14 C3Hf females. The resultant progeny have been used for first generation backcross matings, and six litters have been produced that meet our production requirements. Individuals from these six litters are now being tested for blood catalase levels. Those found to carry the acatalasemic locus (in the heterozygous state) will be used for second generation backcrosses.

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LINKAGE RELATIONSHIP OF THE CATALASE MUTANT IN MICE

Richard C. Dickerman,* Robert N. Feinstein, and Douglas Grahm

PURPOSE AND METHODS

The autosomal mutant "acatalasemia" (Cs^b) in mice discovered by Feinstein and his coworkers⁽¹⁾ was analyzed for its linkage relationship with other mutants in mice. The Cs^b mutant affects the catalase level in blood and all solid tissues of the mouse. The catalase content of the blood was measured by a quantitative perborate assay.⁽²⁾ Feinstein reported several other mutants affecting catalase levels in the mouse

and has presented evidence indicating these other mutants are alleles of Cs^b .⁽¹⁾

The Cs^b mutant is incompletely dominant with the heterozygote (Cs^a/Cs^b), having about 50% of the normal amount of catalase. The homozygous mouse (Cs^b/Cs^b) has little or no detectable catalase.

Mice carrying the Cs^b mutant were crossed with mice containing marker genes and the F_1 offspring were backcrossed to determine random assortment or linkage of the Cs^b mutant and the marker gene. Figure 105 diagrams the various types of crosses made. A recombination value significantly below 50% would indicate linkage of the Cs^b gene and the marker gene.

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Type 1. Used with mutants pink, chinchilla, short ear, and piebald

$$\begin{array}{l} P \quad \frac{p C s^b}{p C s^b} \times \frac{+ C s^a}{+ C s^a} \\ F_1 \text{ backcross} \quad \frac{p C s^b}{+ C s^a} \times \frac{p C s^b}{p C s^b} \end{array}$$

Type 2. Used with steel, ragged, caracul, pintail, twirler, rex, Danforth short tail and varitint

$$\begin{array}{l} P \quad \frac{S l C s^a}{+ C s^a} \times \frac{+ C s^b}{+ C s^b} \\ F_1 \text{ backcross} \quad \frac{S l C s^a}{+ C s^b} \times \frac{+ C s^b}{+ C s^b} \end{array}$$

Type 3. Used with rex, Danforth short tail, and varitint

$$\begin{array}{l} P \quad \frac{R e C s^a}{+ C s^a} \times \frac{+ C s^b}{+ C s^b} \\ F_1 \text{ backcross} \quad \frac{R e C s^a}{+ C s^b} \times \frac{+ C s^a}{+ C s^a} \end{array}$$

Type 4. Used with agouti

$$\begin{array}{l} P \quad \frac{a C s^a}{a C s^a} \times \frac{+ C s^b}{+ C s^b} \\ F_1 \text{ backcross} \quad \frac{a C s^a}{+ C s^b} \times \frac{a C s^a}{a C s^a} \end{array}$$

FIG. 105.—Types of crosses used in recombination analysis

TABLE 33. RECOMBINATION VALUES BETWEEN THE MUTANT CATALASE AND MARKER MUTANTS

Mutant	Linkage group	Parental classes		Recombinant classes		Percent recombination	Chi-square (1:1:1:1), %
Pink	I	67	59	71	64	51.7 ± 3.1	$p = 80$
Chinchilla	I	26	35	33	35	52.7 ± 4.4	$p = 80$
Short ear	II	29	31	39	30	53.5 ± 3.5	$p = 50$
Piebald	III	32	31	29	40	52.3 ± 4.3	$p = 50$
Steel	IV	37	56	43	41	47.5 ± 3.8	$p = 20$
Ragged	V	32	35	32	21	45.0 ± 4.5	$p = 15$
Danforth short tail	V	64	77	38	39	35.3 ± 3.2	$p < 1$
Agouti	V	57	44	9	12	17.2 ± 3.4	$p < 1$
Caracul	VI	41	46	62	44	54.9 ± 3.6	$p = 15$
Rex	VII	37	50	31	43	46.0 ± 3.9	$p = 15$
Pintail	VIII	23	34	33	30	52.5 ± 4.6	$p = 50$
Twirler	XV	25	52	30	47	50.0 ± 4.0 ^(a)	$p < 1$ ^(a)
Varitint	XVI	44	41	40	38	47.9 ± 3.9	$p = 90$
Oligosyndactily	XVIII	26	33	24	27	46.4 ± 4.8	$p = 75$

^(a) Deviation due to lack of *Tw* individuals equally in both parental and recombinant classes.

FIG. 106.—Map of linkage group V

PROGRESS REPORT

Table 33 gives the results obtained with crosses of *Cs^b* to 14 different mutants on 11 different chromosomes. From the table it can be seen that recombination of the *Cs^b* mutant with Danforth short tail and agouti was much less than 50%, indicating linkage with

TABLE 34. EFFECT OF THE SEX OF THE RECOMBINANT PARENT ON THE RECOMBINATION FREQUENCIES

<i>Cs^b-a</i>	17.3% Recombination
♂ Recombinant	5/50 = 10.0
♀ Recombinant	17/77 = 22.1
	$\chi^2 = 2.6 \quad p = 10\%$
<i>Cs^b-Sd</i>	35.3% Recombination
♂ Recombinant	47/124 = 37.9
♀ Recombinant	30/94 = 31.9
	$\chi^2 = 0.87 \quad p = 50\%$
<i>Cs^b-Ra</i>	45.0% Recombination
♂ Recombinant	36/74 = 48.6
♀ Recombinant	18/46 = 39.1
	$\chi^2 = 0.70 \quad p = 50\%$

these two genes which are both in linkage group V. A tentative map showing the relative positions of the genes in linkage group V is given in Figure 106. It appears that the *Cs^b* gene should be quite close to the gene for pallid; test crosses are being run to verify this.

Table 34 gives the data divided as the sex of the recombinant individuals. It can be seen from these data that the sex in which the recombination occurred did not significantly alter the recombination frequencies.

Table 35 presents data demonstrating an interactive effect of the *Cs^b* mutant with three of the dominant markers used. In each case there is a deficiency of the *Cs^b* homozygote when the mutant is also present. Because of these interactions, other crosses using *Cs^b* heterozygotes were utilized for the linkage analysis.

TABLE 35. BIOLOGICAL INTERACTION OF Cs^b/Cs^b WITH THE MUTANTS REX, DANFORTH SHORT TAIL, AND VARITINT

Genotypic classes				Chi-square test for 1:1:1:1 ratio of marker classes	
$Re/+ \ Cs^b/+$ 58 ^(a)	$+/+ \ Cs^b/Cs^b$ 43	$Re/+ \ Cs^b/Cs^b$ 34	$+/+ \ Cs^b/+$ 49	$\chi^2 = 6.26$	$p = <2.5\%$
$Sd/+ \ Cs^b/+$ 53	$+/+ \ Cs^b/Cs^b$ 56	$Sd/+ \ Cs^b/Cs^b$ 21	$+/+ \ Cs^b/+$ 54	$\chi^2 = 13.84$	$p = <1\%$
$Va/+ \ Cs^b/+$ 55	$+/+ \ Cs^b/Cs^b$ 48	$Va/+ \ Cs^b/Cs^b$ 29	$+/+ \ Cs^b/+$ 52	$\chi^2 = 8.04$	$p = <1\%$

^(a) Observed number in indicated class.

CONCLUSION

The mutant Cs^b is located in linkage group V between Danforth short tail and agouti.

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POSSIBLE EXISTENCE OF TWO BLOOD CATALASE ALLELES IN A COLONY OF PEROMYSCUS CRINITUS (CANYON MOUSE)

Robert N. Feinstein, Judith B. Howard, and Joann T. Braun

PURPOSE AND METHODS

During the course of a study of activity levels and heat stability of blood catalase of a variety of species,⁽¹⁾ it was observed that in this Division's colony of canyon mice (*P. crinitus*), blood catalase activity levels clustered around one of three levels, suggesting the existence in the colony of two alleles for blood catalase—a high activity one and a low activity one, with a heterozygote group of intermediate activity. We have, therefore, made blood catalase assays⁽²⁾ on all members of the colony and have attempted to establish matings to determine more definitely whether or not this is indeed a two-allele situation.

PROGRESS REPORT

Table 36 summarizes all data on blood catalase activity levels in the present *P. crinitus* colony. It does

TABLE 36. DISTRIBUTION OF *P. crinitus* BLOOD CATALASE ACTIVITY LEVELS

	No. of animals	Mean ^(a)	Range ^(a)
Low	8	15.5	10-24
Intermediate	17	104.8	70-135
High	7	199.1	175-229

^(a) Values for mean and range are expressed as perborate units/ml whole blood.⁽²⁾

appear that catalase levels can fall naturally into one of three groups, high, intermediate, or low, with no overlap between groups. It will also be noted that the mean catalase activity of the intermediate group is almost exactly the average of the low and the high mean values, a situation shown to exist in known genetic mouse mutants.⁽³⁾

We have attempted to arrange matings to decide more conclusively the genetic basis of this trait in *P. crinitus*. Unfortunately this species does not breed well in captivity, and the only successful matings thus far have been between two members of the intermediate group. If this is indeed a heterozygote group, one would expect progeny of all three sorts, in the ratio one high:two intermediate:one low. Actual results are three high:four intermediate:eight low. All three groups are represented, but the total number of progeny is obviously far too small to give any significance to the ratios.

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THE DOG AS AN EXAMPLE OF ACATALASEMIA OR HYPOCATALASEMIA

Robert N. Feinstein, Joann T. Braun, and Judith B. Howard

PURPOSE AND METHODS

For a variety of studies, it is of interest to have available sources of blood very low in, or completely free of, catalase activity. There are now known acatalasemic or hypocatalasemic humans,^(1, 2) guinea pigs,⁽³⁾ and laboratory mice.⁽⁴⁾ The low catalase members of these species are presumed to be mutants; certainly a far higher blood catalase level is the much commoner occurrence in these species.

Before these low blood catalase sources were available, biologists wanting catalase-free or catalase-low blood would generally select the duck, which had been shown in 1952⁽⁵⁾ to be normally low in this enzyme.

A note in Nature⁽⁶⁾ suggested that dogs fall naturally into three categories of blood catalase activity: low, intermediate, and high. Unfortunately the mode of expression of catalase activity used made it difficult to compare the dog blood catalase activity with that of other species. We, therefore, performed a series of blood catalase assays on dogs, chiefly from this Division's own colony, but also on a few from other sources. Most of the animals were beagles, but there were included one poodle, two German shepherds, and one dog of mixed breed. The breed appeared to be of little importance in determining the blood catalase level. Blood was drawn from the cephalic vein, and catalase was assayed by the perborate method.⁽⁷⁾

TABLE 37. COMPARISON OF BLOOD CATALASE ACTIVITY OF DOG WITH THAT OF OTHER SPECIES

Species	No. tested	Mean ^(a)	Range ^(a)
Dog	83	22.0	0.0-43
Duck	3	1.5	1.4-1.5
<i>Cs^b</i> (acatalasemic) mouse	10	2.6	1.1-4.8
<i>Cs^c</i> (hypocatalasemic) mouse	10	27	18-39
<i>Cs^a</i> (normal) mouse	110	149	105-184
Laboratory rat	3	223	204-238
Guinea pig	2	176	174-178
Rabbit	1	325	—
Man	1	455	—

^(a) Values for mean and range are expressed as perborate units⁽⁷⁾ of catalase activity/ml whole blood. For a description of the *Cs^a*, *Cs^b*, and *Cs^c* mouse strains, see Reference 4.

PROGRESS REPORT

Our general finding is that, although there is some variability among dogs, *all* the dogs we investigated (a total of 87) were to be classified as acatalasemic or hypocatalasemic. Table 37 summarizes the data and compares the blood level of the dog with that of the duck and also with that of a few other representative species.

It has not been possible to establish a genetic pattern to demonstrate the existence of multiple alleles among the dogs. Although high, intermediate, and low catalase groups could be listed, the selection would be purely arbitrary; there is overlap throughout the entire range.

CONCLUSION

Although some variability of catalase activity level of dog blood has been observed, *all* dog bloods appear to be classifiable as acatalasemic or hypocatalasemic. Many dogs appear to be quite as suitable as the commonly used duck for experiments requiring erythrocytes low in catalase activity. It has not been possible to establish a sound genetic pattern of catalase levels amongst the dogs tested.

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EFFECT OF COMPOUNDS RELATED TO AMINOTRIAZOLE ON BLOOD AND LIVER CATALASE. III.

Robert N. Feinstein and Joann T. Braun

PURPOSE AND METHODS

In the 1965 Annual Report of this Division,⁽¹⁾ we described a continuing search for compounds capable of inhibiting the catalase of mouse liver and blood *in vivo*. Although several compounds are known⁽²⁾ which effectively inhibit the catalase of liver and other solid tissues *in vivo*, none has yet been found which will inhibit erythrocyte catalase.

PROGRESS REPORT

Since the last report,⁽¹⁾ the following compounds have been tested:

Purchased from Aldrich Chemical Company:

Rhodanine (2-thioxo-4-thiazolidinone)
4-(2-Thiazolylazo)-resorcinol
Dithiourazole
2-Amino-5-methyl-1,3,4-thiadiazole
2-Amino-5-bromothiazole
2-Amino-4-imino-thiazoline
Furil

Ethyl (2-thenoyl) acetate
2,5-Dimercapto-1,3,4-thiadiazole.

Purchased from K & K Laboratories:

2-Mercaptopyridine
4-Mercaptopyridine

2-Mercaptopyrimidine

3-Aminopyrazole

Purchased from Shell Chemical Co.:

Propane sultone (3-hydroxy-1-propane sulfonic acid sultone)

Purchased from Gallard-Schlesinger Chemical Mfg. Corp.:

2,2'-Furfurylidine azine

2-Furfural hydrazone.

These compounds were injected intraperitoneally at the maximum tolerable (i.e., nonlethal) dose, and blood and liver catalase were assayed one hour later. Of the listed compounds, none significantly decreased blood catalase activity, and only one (4-mercaptopyridine) reduced liver catalase by as much as 20%. The dose of this compound was 100 mg/kg, and liver catalase reduction was exactly 20%.

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GENETICALLY CONTROLLED VARIATIONS IN BENICE-JONES PROTEINS

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PURPOSE AND METHODS

Since the amino acid sequences of proteins are under genetic control, it may be possible to use sequence determinations to deduce the types of genetic changes responsible for the wide variations in Bence-Jones and myeloma proteins. These variations in Bence-Jones proteins, of a particular antigenic type (κ or λ = Type I or II), are currently believed to be restricted mainly to the 107-108 amino acid residues in the amino halves of the protein molecules.^(1, 2) The same generalization can be applied to the corresponding light chains of myeloma proteins.^(3, 4) Moreover, the Fc fragments, representing the carboxyl-terminal

segments of the heavy chains of γ -globulins and myeloma proteins, also do not vary appreciably in a given species.⁽⁵⁻⁷⁾ These observations indicate that the sequences of nucleotides coding for the amino acid constituents of the carboxyl segments of the Bence-Jones and myeloma proteins do not change frequently. The substitutions that have been detected in the C-terminal regions can be explained by point mutations involving the change of a single nucleotide pair. Although a considerable body of information has been accumulated, the major unresolved questions still center on the mechanisms for the wide variability in the nucleotide sequences coding for the amino ends. In addition to the genetic problems, the relations of the variations in sequences to the three-dimensional conformations are also of prime importance. While

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FIG. 107.—Fractionation of polypeptides released from the Dillon κ -chain by cleavage with cyanogen bromide.

beginning to consider these problems from a theoretical point of view, we have also continued to isolate and characterize peptides from three Bence-Jones proteins: one κ -chain (Dillon) and two λ -chains (Hulette-Way and Nevin).

The general methods for fractionation and detection of these peptides are outlined in another report⁽⁸⁾ and in last year's Annual Report.⁽⁹⁾ However, one procedure not previously emphasized played a major role in the more recent progress in the investigation of the Dillon κ -chain. This κ -chain and the Hulette-Way λ -chain contain a single methionine residue, and the peptide bond involving the carbonyl group of this residue can be preferentially cleaved with cyanogen bromide. When the experimental conditions found successful for cleavage of myoglobin⁽¹⁰⁾ resulted in low yields of products from Bence-Jones proteins, the Dillon κ -chain and the Hulette-Way λ -chain were reacted with cyanogen bromide at pH 2 in the presence of a strong denaturing agent, 8 M urea. The polypeptides released from the Dillon κ -chain were initially separated from the urea and cyanogen bromide by gel filtration on a 5- × 165-cm column of Sephadex G-25, (see Figure 107a). These polypeptides were sub-

sequently fractionated on a 5- × 100-cm column of Sephadex G-75. When complete resolution was not achieved with this column, appropriate fractions were selected (see points between diagonal lines in Figure 107b), evaporated to dryness, and placed on a 5- × 169-cm column of the same gel. The components were isolated and subjected to amino acid analysis. The results were interpreted with the aid of the computer program considered in the preceding report.⁽⁸⁾

Similar experiments were performed on the Hulette-Way λ -chain, but the cleavage products could not be separated by the same type of gel filtration. The Nevin λ -chain does not contain methionine and is consequently not suitable for reaction with cyanogen bromide.

PROGRESS REPORT

Dillon κ -chain

The elution patterns for the fractionation of the polypeptides liberated by the action of cyanogen bromide are shown in Figure 107. After removal of the reagents (Figure 107a), the mixture of polypeptides was separated into four components (Figure 107b and c). The amino acid compositions of the last three of these components are presented in Table 38. The

TABLE 38. AMINO ACID COMPOSITIONS OF POLYPEPTIDES ISOLATED AFTER CLEAVAGE OF THE DILLON κ -CHAIN WITH CYANOGEN BROMIDE

Amino acid	No. of residues			
	Polypeptide			Total (3 + 4)
	2	3	4	
Aspartic acid	15.2	11.1	5.04	16
Glutamic acid	26.4	18.1	7.95	26
Glycine	14.6	7.28	7.12	14
Alanine	12.1	7.41	4.77	12
Valine	10.8	10.4	0.96	11
Leucine	18.0	8.58	9.22	18
Isoleucine	5.45	2.09	3.27	5
Serine	32.3	17.8	14.1	32
Homoserine	—	—	0.74	1
Threonine	20.1	10.6	9.06	20
Cystine	4.37	3.77	0.87	5
Methionine	0.48	0.11	0.03	0
Proline	12.9	7.23	5.32	12
Phenylalanine	9.60	6.79	2.65	10
Tyrosine	6.29	4.70	1.68	7
Histidine	2.01	1.84	0.06	2
Lysine	11.7	9.61	2.09	12
Arginine	7.99	3.26	4.16	7
Tryptophan	2.87	2.33	0.73	3
Total number of residues	214	133	80	213

first band consisted of aggregates, the amino acid composition of which could not be interpreted. The composition of the second component was closely similar to that of the unreacted κ -chain, and the results for the third and fourth bands indicated that these were the primary cleavage products.

Since polypeptide 3 did not contain homoserine, the degradation product of methionine in the reaction with cyanogen bromide, it represented the carboxyl-terminal segment of the protein, i.e., it was released by cleavage on the carbonyl side of the methionine residue. Homoserine was a constituent of polypeptide 4, which, therefore, corresponded to the segment from the amino end to, and including, the methionine residue. The combined compositions of polypeptides 3 and 4 were approximately the same as that of the undegraded protein, as represented by component 2.

The division of the protein into two soluble and relatively pure polypeptides makes it possible to interpret the results of the studies of smaller peptides⁽⁹⁾ within more limited and accurate frames of reference. In the segment corresponding to polypeptide 4, for example, there are only six sites (four arginine and two lysine residues) potentially sensitive to the action of the enzyme trypsin. Tryptic peptides containing four (2 *Arg* and 2 *Lys* residues) of these sites have been identified. By combining these results with the composition of polypeptide 4 and the amino acid sequences of other Bence-Jones κ -chains,^(1, 11) we were able to deduce the general characteristics of the two missing smaller fragments. This information was particularly important since the genetically controlled substitutions were relatively numerous in the regions represented by the missing fragments.

The isolation of polypeptide 3 led to more conclusive results on the question of the conservation of sequence in the carboxyl-terminal half of the Dillon κ -chain. Since the composition of the intact protein was significantly different from that of several other κ -chains,⁽⁹⁾ we wondered if the differences were partly manifest in the C-terminal 107–108 residues. Tryptic peptides collectively accounting for 10 *Lys* and 2 *Arg* residues in polypeptide 3 have been detected, and thus far no substitutions have been identified in those peptides assigned to the C-terminal half of the κ -chain. Therefore, with the possible exception of the one fragment still missing, the differences appear to be restricted to the N-terminal half of the protein, as represented by polypeptide 4 and the first 20 residues of polypeptide 3.

Hulette-Way and Nevin λ -chains

One substitution has been detected in position 194 of the carboxyl halves of the Hulette-Way and Nevin

λ -chains. The *Arg* residue in the sequence *Ser.His.Arg* in the Hulette-Way protein is replaced by a *Lys* residue in the Nevin λ -chain. This position corresponds to the *Inv* antigenic locus in the κ -chains, in which valine and leucine are interchanged.⁽⁹⁾ All four residues are apparently on the surface of the protein molecules, since antibodies specific for each of these side-chains can be made.

In view of the close chemical similarity of each group of two interchangeable residues, this specificity is striking. We have begun to consider the problem of the specific binding of antigen to antibody by assuming that a myeloma protein will have structural features analogous to those in a normal γ -globulin molecule. Under this assumption, we can make use of the sequence data for Bence-Jones proteins and for the heavy chains. These sequences can be compared with the sequences of the Fc fragment,⁽⁷⁾ although this part of the molecule is not believed to be involved in the antibody-combining site.⁽⁶⁾ We had initially hoped to approach the problem with the aid of our helical wheels.⁽¹²⁾ However, the immunoglobulins probably do not contain a sufficiently high percentage of helices to make this approach feasible, and alternative procedures will have to be devised.

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COMPUTER ANALYSIS OF CHROMOSOME AREAS OF THE WOOLLY OPOSSUM, *CALUROMYS DERBIANUS*

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PURPOSE AND METHODS

The classification of chromosomes according to groups and subgroups has depended almost entirely on photographs and manual measurements of their

lengths. Classification into major groups, based on centromere position, can readily be made and further identification of individual chromosomes is possible in most cases; but subclassification within these groups

TABLE 39. MEANS AND STANDARD DEVIATIONS OF CHROMOSOME AREA (COMPUTER), MANUAL LENGTH, AND ARM RATIOS OF FEMALE *Caluromys derbianus*

Group ^(a)	Subgroup	Number pairs	Number chromosomes measured	Computer area $\times 10^{-3}$ ^(b)	Length, mm	Ratio, long arm/short arm
SM	A	1	10	4.13 ± 0.74	35.6 ± 2.2	1.75 ± 0.06
SM	B	2	20	3.60 ± 0.59	30.5 ± 2.5	1.63 ± 0.17
M	C	1	10	2.62 ± 0.50	22.1 ± 1.7	1.09 ± 0.11
T	D	1	10	1.62 ± 0.30	13.5 ± 1.1	3.22 ± 0.36
T	E	1	10	1.49 ± 0.27	12.8 ± 1.3	5.27 ± 0.55
T	F (xx) ^(c)	1	10	0.61 ± 0.16	6.5 ± 0.3	5.50 ± 0.33

(a) SM, subtelocentric; M, metacentric; T, telocentric.

(b) $\times 10^{-3}$ computer area units are equal to $3.6 \mu^2$.

(c) (xx), 2 sex chromosomes.

FIG. 108.—Arbitrary chromosome areas (CHLOE) of *Caluromys derbianus* plotted against arbitrary chromosome length (manual). Each point on the line represents measurements of 10 chromosomes from 5 different metaphase spreads. The largest chromosome (subgroup A) is set to equal 1.0.

is very difficult and in some cases impossible by present manual identification procedures.

It is possible to clarify chromosome subgroupings more precisely by computer analysis of chromosome area through the development of a computer program for the optical scanner CHLOE, which calculates the areas of chromosomes.⁽¹⁻³⁾ The classification of the African rat (*Mastomys natalensis*) chromosomes into subgroups, using the computed areas obtained from the CHLOE optical scanner, has been reported previously.^(4, 5) In the present report, a similar analysis has been made of chromosomes of the female woolly opossum, *Caluromys derbianus* ($2n = 14$).^(6, 7)

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The classification of the 12 autosomes and 2 sex chromosomes of *Caluromys derbianus* into 6 subgroups, A-F (Table 39) was made from the computed areas obtained from the digital information from

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FIG. 109.—Photograph (1) is a metaphase spread of 14

the CHLOE scans of negatives, and from manual measurements of arm ratios and lengths of chromosomes in several metaphase spreads. This species has 7 pairs of chromosomes, belonging to 3 distinct groups, determined by centromere position as metacentric, telocentric and subtelocentric. The means and standard deviations of chromosome areas (computed), lengths and arm ratios for 5 metaphase spreads are shown in Table 39. The data show the classification of the 7 pairs of chromosomes in 6 subgroups (A-F), with subgroup B containing 2 pairs. No significant correlation between the three measurements (computed area, measured length, and arm ratios) was found on the 4 individual group B chromosomes, and identification of the homologous pairs within this group remains uncertain. The other pairs were significantly different from each other so that no further grouping was necessary. The two X-chromosomes (subgroup F) of this species were the smallest chromosomes. It was not possible from the three methods of measurements to distinguish any significant difference between the 2 individual X-chromosomes, as was found for *Mastomys natalensis*.⁽¹⁾ There is a close correlation between the computer determined areas and manually measured lengths as seen in Figure 108. The largest chromosomes (subgroup A) are the longest, and the shortest chromosomes (subgroup F, XX) have the smallest area; the other chromosomes fall between these two groups.

Chromosomes of *Caluromys derbianus* are shown in Figure 109. Number (1) represents the metaphase spread on 35-mm film (negative) which is the input for the CHLOE scanner; (2) shows the chromosomes as reconstructed from the digitized information obtained from the scan, and lines connecting computer determined homologues; (3) is the karyotype and subgrouping of chromosomes as determined by the computer from area measurements and six other moment invariants incorporated into the computations; and (4) is the manual karyotyping (pairing), based on visual selection of the chromosomes from photographs, (arm ratios and lengths). The relative lengths are consistent with those described previously for this species.

CONCLUSION

The classification and grouping of chromosomes of other mammalian species will be made. X-chromo-

somes of *Caluromys derbianus* representing the input data for the optical scanner (CHLOE); (2) chromosomes reconstructed from the digital information and connecting lines from individual chromosomes to their homologous pairs as determined by the computer; (3) karyotype and subgrouping of chromosomes as determined by the computer; (4) manual karyotype and grouping of the chromosomes.

somes will be analyzed isotopically and histologically, and these properties will be compared with information obtained from computer analysis.

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ACATALASEMIC AND HYPOCATALASEMIC MOUSE MUTANTS. VI. POSSIBLE RELATIONSHIP OF CATALASE ACTIVITY TO CELL AND ANIMAL AGE

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PURPOSE AND METHODS

H. Aebi's group in Switzerland, studying human acatalasemia, found that the trace of catalase activity in acatalasemic blood was very largely traceable to the reticulocyte fraction.⁽¹⁾ Based on this observation, Aebi generalized that young cells in acatalasemic people or mice might be more active catalatically than older cells. He suggested the specific experiment of partially hepatectomizing acatalasemic mice, to see whether or not the newly regenerated hepatic tissue might be higher in catalase activity than nonregenerating liver.

We have tested Aebi's generalization in the way he suggested, and also in several other ways:

- a) Measuring catalase activity of acatalasemic blood before and after bleeding, in the expectation of a pulse of erythrocytes showing an increased catalase activity.
- b) Measuring liver catalase activity before and after partial hepatectomy, as noted above.
- c) Measuring whole-body catalase activity of mouse embryos, young, and adults, based on the consideration that embryos presumably contain a higher proportion of newly formed cells than do young mice, which in turn contain a higher proportion of newly formed cells than do adult animals.
- d) Measuring liver catalase activity in mice of various ages, the basis being the same as (c) above, but with the advantage of greater homogeneity due to use of a single tissue.

TABLE 40. EFFECT OF BLOOD LOSS ON BLOOD CATALASE ACTIVITY OF ACATALASEMIC MICE

	First sample	Second sample
Catalase (PU/ml) ^(a)	2.2	1.6
Hemoglobin (g/100 ml)	14.6	12.5
Catalase (PU/g Hb)	15.1	12.8

^(a) PU = perborate units of catalase activity.⁽³⁾ Each datum is the mean value of 10 mice. Blood drawn was approximately 0.2 ml, which is estimated to be about 10% of the total blood supply of these mice. Six hours elapsed between drawing of the two blood samples.

Bleeding of mice was by the orbital sinus technique.⁽²⁾ Catalase assays were performed by the perborate method.⁽³⁾ Hemoglobin was determined as cyanmethemoglobin.⁽⁴⁾ Mice used were the normal, wild type (*Cs^a*) and the acatalasemic mutant strain (*Cs^b*) described earlier.⁽⁵⁾

PROGRESS REPORT

In a preliminary experiment, there was a suggestion that at 6 hr after a blood-letting, but not after 24 or 48 hr, there was an increased catalase activity per gram of hemoglobin. In a more careful following experiment, however, this suggestion was not confirmed. Table 40 shows the results of this second experiment.

Table 41 provides the results of a partial hepatectomy experiment, as mentioned earlier. Surprisingly, there is a clear diminution of liver catalase activity in regenerating liver, rather than the postulated increase. This finding is at least partially explained by the observation that even sham surgery, i.e., laparotomy without hepatectomy, sharply decreases liver catalase activity. This is consonant with the findings

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TABLE 41. EFFECT OF PARTIAL HEPATECTOMY ON LIVER CATALASE OF ACATALASEMIC MICE

Time between partial hepatectomy and sacrifice, hr	Catalase activity		Change, %
	First sample	Second sample	
24	324	215	-34
36	417	250	-40
48	316	203	-36

Each datum is the mean of values for three mice. Catalase activity is expressed as perborate units per gram liver.⁽³⁾ At hepatectomy, approximately one third of the total liver was removed.

TABLE 42. EFFECT OF AGE ON WHOLE-BODY AND LIVER CATALASE

Days post-conception	Whole-body catalase			Liver catalase		
	Normal	Acatalasemic	Ratio Norm./Acat.	Normal	Acatalasemic	Ratio Norm./Acat.
10	22	5.6	3.9			
15	14	4.7	3.0			
20	38	12	3.2			
21				528	97	5.4
25	37	13	2.8	527	142	3.7
30	44	18	2.4	709	213	3.3
40	42	13	3.2	1070	227	4.7
55	97	24	4.0	1610	386	4.2

To establish conception date, males and females were provided only 24 hours' contact. Each datum is mean value of three separate animals. Different mice were used for liver and for whole-body catalase, so that the whole-body activities include the hepatic tissue. Data provided are perborate units of catalase activity per gram whole body or liver.⁽³⁾

of Nishimura et al.⁽⁶⁾ that such traumata as the subcutaneous implantation of tale will depress liver catalase activity.

Table 42 lists whole-body and liver catalase of embryo and free-living mice of various ages. Both

normal and acatalasemic animals are included. It is evident that in both normal and acatalasemic mice, the catalase activity of the liver and of the whole body increases with advancing age. However, the ratio of the two strains remains approximately constant; aging of the acatalasemic animals increases catalase no faster than aging of normal mice.

CONCLUSIONS

Evidence has been obtained that withdrawal of blood, which might be expected to produce a pulse of reticulocyte-rich cells, has no effect on blood catalase activity levels, based on hemoglobin.

Partial hepatectomy of acatalasemic mice causes a considerable decrease in catalase of the remaining hepatic tissue. This may be largely due simply to the trauma of the surgery, without regard to the accrual of newly formed hepatic cells in the regenerating liver.

Embryos and young free-living mice, both acatalasemic and normal, show increases with age in catalase activity of liver and of whole body. The ratio of activities of the two strains of mice, however, remains fairly constant.

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Microbial Genetics

THE GROWTH-DUPLICATION CYCLE

Herbert E. Kubitschek

INTRODUCTION

Although the events that occur during the cell growth and division cycle can be outlined in a broad and general way, our knowledge of the controls and of explicit details of large parts of the cycle are scanty,

at best. If different kinds of cells employ common facets in their modes of control of cell growth, these have not been identified. Indeed, growth studies of single cells have shown marked differences for different organisms. Furthermore, although the cell cycle is

divided for convenience into several periods (G_1 , S , G_2 , D), the events triggering the transition from one period to the next can only be surmised.

The relatively recent advent of improved synchronous culture techniques promises a better understanding of the cell cycle, an opinion attested to by the great increase recently in the number of investigators applying these techniques.

As for the pattern of cell growth in bacteria, there has been some recent concurrence that these, at least, should grow in an essentially exponential manner during the division cycle. This viewpoint seems to stem mainly from the observation that the synthesis of protein proceeds exponentially in synchronous cultures (see Ref. 1, for example) and from the rationale that the amount of protein synthesized in a cell should increase exponentially if ribosomes are made continuously and if protein synthesis is proportional to ribosomal content (see Ref. 2, for example). But this viewpoint also assumes cell growth is accounted for

completely by macromolecular synthesis. This is not so, since all growing cells also contain pools of compounds of low molecular weight required for macromolecular synthesis.

Furthermore, the experiments in the following series of reports do not support the theory of exponential bacterial growth during the cycle. This series concerns the synchrony technique used in these experiments, the observations of linear growth in bacteria and other cells, and a possible explanation for a mechanism of linear growth that was subjected to several tests. These reports are presented here in series (I through VI) to facilitate their discussion and interpretation.

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THE GROWTH-DUPLICATION CYCLE. I. SYNCHRONOUS BACTERIAL CULTURES

Herbert E. Kubitschek

PURPOSE AND METHODS

Study of the growth-duplication cycle and its control in bacteria requires synchronized cultures. Of the available techniques to obtain synchrony, the method of Mitchison and Vincent⁽¹⁾ appears to be the most appropriate for this purpose. Cells with a narrow range of properties are selected from a steady-state population in balanced growth. The culture is concentrated by centrifugation, then layered upon a sucrose density gradient and centrifuged again for a brief period (velocity centrifugation). Because large cells settle more rapidly than small cells, the original layer is spread into a broad band with smaller cells near the top of the band and larger cells near the bottom. When Mitchison and Vincent examined the smaller cells from the top of this band, they found that their division times were rather synchronous.

Their results suggested that the Mitchison-Vincent technique might provide us with a highly-synchronized population of cells in the same steady state established for the parent culture. It was necessary first, however, to establish as far as possible the conditions that eliminate any perturbations from steady-state growth.

PROGRESS REPORT

We tested this technique with several strains of *Escherichia coli* under several different growth conditions. Quantitative details of the gradient technique and the size resolution obtainable have been described.⁽²⁾ The best resolution was obtained when bacteria were concentrated into pellets preparatory to layering. Resolution was poorer when bacteria adhered to the wall of the centrifuge tube and failed to form a pellet; presumably, steady-state growth conditions are altered when freely growing bacteria interact with a surface. In some cases, adhesion to the wall of the tube was eliminated by the addition of sucrose, usually added to the growth medium to give a concentration of about 2%.

Filtration methods to concentrate cells usually gave somewhat inferior results to those obtained when cells were concentrated by centrifugation. Synchrony was markedly reduced during the first division cycle when cellulose acetate (Millipore) filters were used. However, good synchrony was obtained during the first division cycle with cellulose nitrate filters (Bac-T-Flex, Schleicher and Schuell), but synchrony was much poorer during the second division cycle.

FIG. 110.—Cell volumes and counts for a synchronous culture of *E. coli*, THU. Cultures were grown in a minimal medium (M9) containing salts and sodium acetate. (a) and (b),

To eliminate a slight lag in growth, of the kind shown in Figure 110, it was often necessary to maintain the following conditions: (1) carry out all manipulations at the temperature of the parent culture, 37°C, (2) return the synchronized cell fraction to the original growth medium, rather than to fresh growth medium, and (3) grow cultures in the presence of sucrose (2%) to reduce excursions in osmotic pressure during velocity sedimentation in the sucrose gradient. Under these circumstances, synchronous cultures have been obtained which show no detectable lag in growth, and corresponding control cultures which show no detectable change in rate of division.

CONCLUSION

Synchronous cultures were obtained with a selection technique that gave no detectable perturbation in cell growth or division rate. Cultures of this kind can be used to study processes of growth and division during the cell cycle. The method is useful also for many other kinds of studies that depend upon cell cycle, including mutation or biochemical properties.

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synchronous cultures; (c), control culture. The entire band was removed from the sucrose gradient, cells were mixed, and a fraction of these were inoculated into the same growth medium as that used for the synchronous culture. The vertical dashed lines indicate the period required for the doubling of cell numbers. In both (a) and (c) the initial point lies above the line fitted through the data, as would be expected for a brief lag in cell growth and division.

THE GROWTH-DUPLICATION CYCLE. II. LINEAR CELL GROWTH IN BACTERIA

Herbert E. Kubitschek

PURPOSE AND METHODS

A knowledge of the kinetics of growth of cells is valuable for elucidating the mechanisms controlling cell growth and duplication. Although many past attempts have been made to measure the growth rates of individual bacterial cells microscopically, they were ineffective because of the limiting optical resolution with visible light. An alternative approach, presented here, is to measure the mean rate of increase of cell size in synchronous bacterial cultures through the use of a

Coulter counter-analyzer system. Synchrony was established in these cultures by the Mitchison-Vincent technique, described in Report I of this series.

PROGRESS REPORT

So that the pattern of growth might be examined at several rates of division, cultures of the bacterium *Escherichia coli*, strains THU and WP2-HCR⁻, were grown in three different media: (1) nutrient broth, or minimal media (M9 salts) containing either (2) glu-

FIG. 111.—Average cell count and mean cell volume for a synchronous broth culture of *E. coli* THU. Cell concentrations are about $10^4 \times$ cell count in all figures.

FIG. 112.—Mean cell volume for a synchronous glucose culture of *E. coli* THU. This figure shows the result of growing the synchronous culture in fresh medium: growth rates were reduced but remained linear. ●, fresh medium; ×, filtered parent medium.

cose, 0.1% or (3) sodium acetate, 0.25%, as the sole source of carbon. Required growth factors were also added, and frequently a nonutilizable sugar, sucrose, was added to a final concentration of 2% to increase osmotic pressure. The corresponding doubling times of cultures in these three media were about 20, 60, and 140 min. In each case mean cell volumes increased linearly over the first synchronous division cycle (Figures 111–113). Figure 114 presents the observed size distributions from which the mean cell volumes were calculated for Figure 111. From Figure 114, it can be seen that the degree of synchrony is reduced with each successive cell division.

Linear growth is not limited to synchronous cultures established from the smallest cells in the parental population: growth is also linear for cultures with larger initial mean cell volumes (Figure 115). Furthermore, it may be seen from this figure that essentially the same growth rates were found for each of the three different sister synchronous cultures in this experiment.

A third kind of experiment was undertaken to answer the following question: Is the observation of a linear increase in mean cell volume an artifact arising from the excessive loss of the largest cells from each cycle by division? If it is, the mean cell volume of the undivided cells would be reduced, and the actual dependence of growth rate upon cell volume might be

FIG. 113.—Average cell count and mean cell volume for a synchronous acetate culture of *E. coli* WP2-HCR⁻.

obscured. Were this true, the width of the distribution should then become markedly reduced at the end of the cycle. But these widths remain relatively constant (see Figure 114).

In order to test in another way for the possibility that linear growth is a division artifact, we took advantage of the fact that cell size depends upon cell growth rate; mean cell volumes for broth cultures are about four times as large as those in acetate media. It is possible, therefore, to prevent cell division as cells increase fourfold in volume by adding nutrient broth and glucose to an acetate culture. During this period,

FIG. 114.—Observed cell size distributions for the culture of Figure 113. The triangles at the base of each distribution indicate the value of the mean cell volume calculated for each peak.

FIG. 115.—Independence of growth rate and cell size. Three synchronous cultures were initiated from cell samples of different mean cell sizes, all selected from the same parent culture of *E. coli* WP2-HCR⁻ growing in acetate medium. The initial cell size distributions are shown to the left of the volume growth curves. The mean values, X, for the sample with the largest cells were calculated only for the undivided cell fraction, that is, the largest of the two peaks constituting the distribution.

cell counts remain constant. When this experiment was performed, mean cell volumes again increased linearly, Figure 116, during the period taken to double mean cell volume. Thus, linearity was observed for cultures in which cells were prevented from dividing, as well as in cultures in which there was no apparent deviation from steady-state kinetics.

If individual cell densities do not change significantly during the cell cycle, the linear increase in mean cell volume should also reflect a linear increase in cell mass. To test this, cells from a culture of *E. coli* THU grown in the minimal-salts-glucose medium were separated by size in a sucrose gradient, and the value of optical density was compared with the mean cell volume for each sample. Optical density is known to be proportional to cell mass in cultures of growing bacteria, so a strict proportionality between optical density and mean cell volume would be reflected in a proportionality between optical density and mean cell volume. This relationship was demonstrated, Figure 117.

In a further attempt to measure relative cell densities, cells from a steady-state culture were centrifuged in linear density gradients of bovine serum albumin, and in Ficoll (a polysaccharide of high molecular weight, approximately 400,000). The range of cell densities was estimated from visual determinations of band width. These band widths were extremely narrow, corresponding to a density range of about 10^{-3}

FIG. 116.—Cell size distributions and mean volumes for a nondividing culture of *E. coli* WP2-HCR⁻. A synchronous culture was obtained from a steady-state parent culture growing in acetate medium and shifted to more rapid growth conditions by the addition of glucose and nutrient broth. Average cell count remained constant for a period of two successive doublings of cell volume.

g/cm³. This result and the measurement of optical density provide evidence that cell mass also increases

FIG. 117.—Values of optical density per cell as a function of mean cell volume in a glucose culture of *E. coli* THU. Cell size distributions are shown for the first and last samples to illustrate the degree of dispersion.

in an essentially linear manner during the period of linear cell volume increase.

CONCLUSION

Cell volume and cell mass increased in an essentially linear manner for most of the growth-duplication cycle in steady-state cultures of *E. coli*.

THE GROWTH-DUPLICATION CYCLE. III. EVIDENCE THAT LINEAR GROWTH IS THE FUNDAMENTAL FORM OF CELL GROWTH AND ITS IMPLICATIONS

Herbert E. Kubitschek

PURPOSE AND METHODS

Does linear growth represent a fundamental growth pattern? This question bears upon the generality of the control mechanisms in cell growth. A partial answer can be obtained by studying the literature on growth patterns that have been found for single cells large enough to be measured with microscopic and/or electronic precision.

PROGRESS REPORT

Linear growth has been observed in some, but not all, eukaryotic cells. *Paramecia* and *Amoeba* did not grow at a constant rate during their cell cycles; however, these cells were cultured upon other orga-

nisms.^(1, 2) If we rule out cells that ingest other organisms and their macromolecules, the finding of linear growth has been rather consistent. Linear growth was observed for *Tetrahymena* by measurements of cell respiration rates⁽³⁾ and cell volumes.⁽⁴⁾ A linear increase in mean cell volumes has also been observed for synchronized cultures of hamster cells.⁽⁵⁾ Periods of linear growth have been observed even for cultures in entrained synchrony, as described for *Euglena*.⁽⁶⁾ The most complete measurements for any organism, are those compiled by Mitchison and his collaborators (see reference 7) for yeast. Using interference microscopy, they showed that cell mass increased linearly over most of the cycle, although growth rates changed

for a brief period of time encompassing cell division. On the other hand, Mitchison and his collaborators also found that macromolecular mass increased in an essentially exponential fashion over most of the yeast cell cycle.

To explain these findings, Mitchison and Wilbur⁽⁸⁾ postulated the existence of a pool of low molecular weight substances that would first increase and later decrease during the cycle. In this manner, the overall rate of mass increase could be constant even though macromolecular mass increased exponentially. The existence of this regularly varying pool of substances in yeast was demonstrated directly by measurements of cell mass with and without extraction of this soluble pool.⁽⁷⁾

Some studies of macromolecular synthesis in synchronous cultures of bacteria have given results parallel to those for yeast,^(9, 10) and these are also supported by more recent measurements using newer techniques.^(11, 12) In all of these, the rate of synthesis of RNA and of protein increased during the cell cycle. Thus we may expect to find low molecular weight pools in bacteria that vary in the same fashion as those found for yeast. In this case, just as Mitchison suggested for yeast,⁽⁷⁾ the linear mass increase would be due to the constant rate of accumulation of nutrients.

Constancy of accumulation of nutrients would seem to require constancy of uptake. If constancy of growth rate is due to constancy of uptake, the functional binding or accumulation sites involved in the uptake of exogenous growth factors may be expected to be limiting and constant in number throughout the period of linear growth. Furthermore, this overall constancy might then be due to the presence of constant numbers of each of the many different kinds of accumulation sites needed for the major components taken up by the cell, such as amino acids, sugars, and inorganic ions. If these predictions are valid, cells in balanced cultures would take up all of the major low molecular weight growth factors at a constant rate during their period of linear growth.

CONCLUSION

For cells growing on low molecular weight compounds the fundamental growth pattern appears to be

linear. Linear growth would occur if each of the different kinds of functional accumulation or binding sites involved in the uptake of growth factors were present in constant amount during the linear portion of the growth-duplication cycle. Cell uptake would then be essentially constant for all of these factors during the period of linear growth.

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THE GROWTH-DUPLICATION CYCLE. IV. CONSTANCY OF UPTAKE OF LABELED GROWTH FACTORS

Herbert E. Kubitschek

PURPOSE AND METHODS

Linear cell growth in steady-state cultures implies a constant uptake of all major growth factors. The pattern of uptake during the cell cycle can be tested by exposing cultures to labeled growth factors for a brief period, then by separating the cells by size on a sucrose density gradient and measuring the amount of label incorporated per cell. If uptake is constant the radioactivity per cell will be essentially independent of mean cell size.

FIG. 118.—The rate of uptake of ^{14}C -glucose as a function of mean cell size in culture of *E. coli* in the exponential growth phase WP2-HCR⁻. In this and other figures $\bar{V}_s$ is the mean volume of the cells in the sample, and $\bar{V}_b$ is the mean cell volume at birth. The calculation of $\bar{V}_b$ has been described earlier.⁽¹⁾ The average coefficient of variation of cell volume, $\overline{CV}$, was approximately 0.18.

FIG. 119.—The rate of uptake of ^{14}C -leucine as a function of mean cell volume in an acetate culture of *E. coli* B_{s-1} in the exponential growth phase.

For these experiments, two sugars, two amino acids, and two inorganic ions (^{14}C -acetate, ^{14}C -glucose, ^{14}C -glycine, ^{14}C -leucine, ^{32}P -phosphate, and ^{35}S -sulfate) in labeled form were added to cultures of *E. coli* growing in the exponential phase in a minimal medium containing either acetate or glucose as the sole source of utilizable carbon. The label was added for a period of 5% of the doubling time and the cells were then killed by the addition of formalin (10%). The formalin-treated culture was filtered, washed with unlabeled growth medium, and layered upon a sucrose density gradient for size separation of the cells. Each sample was divided into two portions, to determine (1) sample radioactivity in counts per minute by the use of a scintillation counter, and (2) cell count and size distribution by the use of a Coulter counter. Detailed techniques have been presented earlier.⁽¹⁾

PROGRESS REPORT

In each case, the average amount of labeled material per cell was relatively independent of the mean cell size in the sample. Results are shown in Figures 118–120 for one compound of each kind of growth factor.

CONCLUSION

The average rates of uptake per cell during the cell cycle were measured for two representatives of each major class of low molecular weight compounds involved in the growth of cells: sugars, amino acids, and

FIG. 120.—The rate of uptake of ^{35}S as a function of mean cell volume in an acetate culture of *E. coli* B_{s-1} in the exponential growth phase. The mean sample dispersion is shown by the horizontal line segment.

inorganic ions. Of all of these compounds, uptake was essentially independent of cell size during the growth-duplication cycle. These results support other findings of linear cell growth (Report II of this series) and also support the hypothesis that uptake of each major growth factor is limited by the presence of a constant number of functional accumulation sites during the

greater part of each cell cycle (Report III of this series).

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THE GROWTH-DUPLICATION CYCLE. V. ABSENCE OF THE REQUIREMENT FOR A SPECIFIC ENERGY SOURCE FOR UPTAKE OF GROWTH FACTORS

Herbert E. Kubitschek

PURPOSE AND METHODS

Reports I to IV in this series support an interpretation of linear cell growth in relation to the presence of

FIG. 121.—Failure of a shift to more utilizable energy source to affect growth rate during the first synchronous division. Two synchronous cultures were established for a parental culture of *E. coli* WP2-HCR⁻ growing in acetate medium. One of these contained only acetate as the energy source, while the other contained glucose in addition to the acetate. ●, acetate only; ×, acetate + glucose.

a constant number of functional accumulation sites throughout most of the cell growth and division cycle. If this interpretation of linear growth is valid, new accumulation sites are not synthesized during the period of linear growth, and uptake of growth factors may be expected to be independent of the specific energy source if the supply of energy is not limited. This hypothesis provides a further test of the interpretation of linear growth by shifting bacterial culture conditions from the presence of an energy source that is used relatively inefficiently (e.g., acetate) to one that is used more efficiently (e.g., glucose).

PROGRESS REPORT

A culture of *E. coli* WP2-HCR⁻ was grown to its exponential phase in acetate-minimal salts medium containing 1% sucrose, a nonutilizable sugar added to increase osmotic pressure. A synchronous subculture was obtained and divided into two fractions. The first fraction was reinoculated into filtered parent cell growth medium. The second fraction was reinoculated into a portion of the same medium, except that glucose (a utilized sugar) was added to give a final concentration of 2%. Both cultures grew synchronously, with no significant difference in their volume growth rate, Figure 121.

CONCLUSION

Linear growth of *E. coli* WP2-HCR⁻ is not measurably affected by the substitution of a more efficient energy source (glucose) for a poorly used energy source (acetate). This result supports the concept that new functional accumulation sites are not produced by this strain of *E. coli* during linear growth.

THE GROWTH-DUPLICATION CYCLE. VI. CONTROL OF GROWTH AND CELL DIVISION

Herbert E. Kubitschek

PURPOSE AND METHODS

The fact that cell growth is linear while macromolecules accumulate exponentially requires the operation of at least two sets of controls of the cell cycle: (1) those which control growth, and (2) those which control cell division. When are these controls applied during the cycle? Their timing can be investigated by effects of changes in the growth media.

A synchronous subpopulation was selected from a culture of *E. coli* THU, grown to the exponential phase in acetate medium (containing 2% sucrose). This synchronous subpopulation was divided into three cultures. Nutrient broth was added to the first at a final concentration of 5%. Glucose was added to the second at a final concentration of 2%. The third was undisturbed and kept as a synchronous acetate control culture. An asynchronous control also was prepared by extracting all of the remaining cells from the gradient, mixing them, and introducing a small fraction of this

FIG. 122.—Average cell counts in metabolically-shifted cultures and in control cultures. Three synchronous cultures and an asynchronous control were prepared from a parent culture of *E. coli* THU growing in the exponential phase in acetate medium. Two of these were shifted up by the addition of nutrient broth or glucose. Synchronous cultures: $\times$, broth added; $\bullet$, glucose added; $\circ$, no additive; Δ asynchronous control.

FIG. 123.—Mean cell volumes for the cultures in Figure 122. For clarity, ordinate values for the broth culture, $\times$, have been plotted at $0.2 \mu^3$ above observed values.

mixture into a filtered portion of the parent culture growth medium.

PROGRESS REPORT

Cell counts and cell sizes were measured for at least two successive synchronous divisions in each culture. In all three synchronous cultures, cell counts began to increase at about the same time (Figure 122). This figure demonstrates little or no effect of the addition of glucose or nutrient broth on the onset of cell division, although the steady state of cell division in such cultures is increased by factors of about 3 and 6 respectively. The duration of the second broth cycle was reduced, however. From these results we conclude that the duration of the interdivision cycle is established either very early in each division cycle or late in the cycle preceding it.

The volume growth rate (linear volume increase) of the culture to which glucose was added was indistinguishable from that of the control culture with acetate alone, Figure 123. The growth rate in the broth culture was appreciably more rapid, however, and also failed to maintain linearity toward the end of its cycle. This increased growth rate can be attributed to the presence of growth factors in nutrient broth that are not available to bacteria in minimal salts media, and otherwise unused accumulation sites may now be avail-

able for the uptake of these growth factors, this allowing an increase in rate of total uptake. The further increase in rate of growth near the end of the cycle in broth cultures might be explained as resulting from the production of new functional accumulation sites. In this case, these sites are produced toward the end of each cell division cycle, rather than at its beginning.

From general considerations, the control of cell generation time might also be established during the final portion of each division cycle. If controls are established at this time, cell characteristics can be modified

during this period. Experiments are in progress to test this interpretation of cell growth controls.

CONCLUSION

In steady-state cultures, the control of cell growth and generation time appear to be established either near or during the time of cell division. Evidence is presented that the bacterium's capacity for growth during any division cycle is established during the final portion of the preceding division.

MUTAGENESIS IN BACTERIA BY VISIBLE LIGHT

Robert B. Webb and Mylan M. Malina*

PURPOSE AND METHODS

Photodynamic mutagenesis in the presence of added acridine dyes has been reported in chemostat cultures of bacteria.⁽¹⁾ During subsequent studies of dye-sensitized visible light mutagenesis, we found that visible light alone (without an added dye) is mutagenic.⁽²⁾

The purpose of this study is to define the conditions for the induction of mutations with visible light, to identify the chromophore(s) responsible for the effect, and to obtain information concerning the nature of the mutational event in DNA. The basic techniques used have been described elsewhere.⁽¹⁻⁴⁾ Continuous cultures (chemostat) of *Escherichia coli* B/r/1, try⁻ or *E. coli* WP2-hcr⁻ were grown in a minimal medium (M9) containing an excess of tryptophan and a limiting concentration of glucose (100 mg/l with air; 300 mg/l with nitrogen). These conditions maintained cell concentrations of 1 to 3×10^8 cells/ml in balanced growth. Turbidostat cultures were grown in the same medium except for the presence of an excess of both tryptophan and glucose (250 mg/l). The turbidostat technique has been described.⁽⁵⁾ The primary mutation (to resistance to the bacteriophage T5) was assayed by plating cells in the presence of excess phage T5 on nutrient agar (Difco) supplemented with iron. All colony counts were made after a 24-hr incubation at 37°C. The growth vessels (chemostats or turbidostats) were placed in small, light-tight incubators designed for these experiments, or were individually isolated in a constant temperature room. The temperature of the growth vessels was maintained at 37°C.

Radiant energy was supplied by several different sources. These included (1) lamps containing two 4-

watt "cool white" fluorescent bulbs, (2) lamps containing two 4-watt "black light" fluorescent bulbs with a mean emission of about 355 nm, (3) a monochromator (Bausch and Lomb) with a super pressure Hg-vapor lamp, (4) a slide projector, containing a 400-watt lamp rated at 200 hr, and (5) a small low pressure Hg-vapor lamp (Penray) with a pin-hole shield. Corning "sharp-cut" filters were used with the fluorescent lamps to obtain preliminary information about the action spectrum of visible light mutagenesis. Interference filters (Baird-Atomic B1) were used with the projector. The optical characteristics of each filter were measured with a Cary recording spectrophotometer. The irradiance (intensity) for each condition was measured with a Schwarz vacuum thermopile and a Keithley 150B microvoltmeter. The thermopile was standardized against a National Bureau of Standards lamp. Measurements of irradiance were reproducible within $\pm 1\%$.

PROGRESS REPORT

Mutation rates greatly in excess of the spontaneous rate (dark) were produced by continuous intensities of visible light (408 ± 4 nm) of 75 ergs/mm²/sec (Table 43). The mutation rate in the dark was 7.3 mutants/10⁹ cells/hr, whereas at an irradiance of 75 ergs/mm²/sec it was 50 mutants per 10⁸ cells/hr, more than six times the dark mutation rate. For unfiltered fluorescent lamps, mutation rates were proportional to irradiance over the range from 125 to 965 ergs/mm²/sec.⁽²⁾

Individual chemostat experiments showing relative effects of different wavelengths of radiant energy are shown in Figure 124. Accumulation of mutants appears to be linear in each case, indicating the absence of selection either for or against the mutants in these experiments. From these and many other experiments,

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TABLE 43. MUTATION RATES, DOSE RATES, LIGHT SOURCES, AND FILTERS FOR CHEMOSTAT EXPERIMENTS USED IN FIGURE 125

Lamp ^(a)	Gas	Filter	Peak wavelength, nm	Half band-width, nm	37% Trans., nm	Ergs/mm ² /sec	Mutants/10 ⁹ cells/hr ^(b)	Mutant/10 ⁹ cells/10 ⁶ ergs/mm ²
<i>E. coli</i> B/r/1, try ⁻								
Dark	Air	none	—	—	—	—	7.3 ^(c)	—
FL	Air	2412	—	—	613	410	-2.5	-2.0
FL	Air	3384	—	—	498	691	3.8	1.6
FL	Air	3385	—	—	477	719	25.8	8.1
FL	Air	3387	—	—	441	739	45.8	17.2
FL	Air	3389	—	—	424	791	86.3	30.3
FL	Air	none	—	—	—	990	97.0	27.2
FL	Air	5850	410	65	—	110	31.3	78.0
Proj	Air	B1	408	6	—	75	50.0	182.0
BL	Air	none	355	60	—	110	51.3	128.0
BL	Air	7380	368	25	359	34	30.8	251.0
Hg-SP	Air	none	315	8	—	34	94.2	772.0
Hg-LP	Air	none	254	—	—	0.05	161.0	89 × 10 ⁴
Dark	N ₂	none	—	—	—	—	4.5 ^(c)	—
FL	N ₂	2412	—	—	613	395	-0.8	-0.6
FL	N ₂	3384	—	—	498	691	1.1	0.4
FL	N ₂	3389	—	—	424	791	-0.5	-0.2
FL	N ₂	none	—	—	—	573	-1.4	-0.7
BL	N ₂	none	355	60	—	96	7.5	20.8
BL	N ₂	7380	368	25	355	45	-1.1	-0.7
<i>E. coli</i> WP2-her ⁻								
Dark	Air	none	—	—	—	—	7.0 ^(c)	—
FL	Air	3384	—	—	498	621	2.8	1.2
FL	Air	3389	—	—	424	647	15.8	6.8
FL	Air	none	—	—	—	573	123.0	60.0
FL	N ₂	3389	—	—	424	640	-1.9	-0.8
BL	Air	none	355	60	—	44	145.0	930.0
BL	N ₂	none	355	60	—	148	290.0	542.0
BL	N ₂	none	355	60	—	96	166.0	482.0

^(a) FL = cool white fluorescent lamps; Proj = projector with 400-watt tungsten lamp; Hg-SP = super pressure mercury lamp in monochromator; Hg-LP = low pressure mercury lamp; and BL = long ultraviolet fluorescent lamps (black light).

^(b) Mutation rates with dark mutation rate (7.3 mutants/10⁹ cells/hr in air; 4.5 mutants/10⁹ cells/hr in 95% N₂, 5% CO₂) subtracted. Each value is the average of at least two independent experiments.

^(c) Spontaneous (dark) mutation rates.

selection has been ruled out as a significant factor in the results reported here. The mean delay, the mean time between the application of mutagen and the beginning of mutant accumulation in the chemostat, was shorter for short and long ultraviolet light (3 generations) than for visible light (5 generations).

Action spectrum data for the induction of phage T5 resistance in *E. coli* from visible light fluorescent lamps with sharp-cut filters, black light fluorescent lamps with sharp-cut filters, and Hg-lamps for shorter wavelengths are shown in Figure 125 and Table 43. In Figure 125, each of the values of efficiency was calculated from the difference between two mutation rates and the respective irradiances obtained with two closely spaced sharp-cut filters with the fluorescent lamps. The mutation rates given in Table 43 (each value the

average of 2 to 5 separate experiments) had standard errors of 5 to 12% with respect to reproducibility. Relative dosimetric measurements, however, were reproducible with an accuracy of $\pm 1\%$. Although precise data will await completion of the action spectrum with single wavelength sources, now in progress, certain significant observations can be made from our present data (Figure 125 and Table 43). Wavelengths above 600 nm are ineffective; wavelengths between 500 and 600 nm are very slightly, but significantly, effective; wavelengths between 400 and 500 nm are highly effective; wavelengths between 390 and 410 nm may be less effective than those immediately below and above; wavelengths between 340 and 380 nm (long ultraviolet radiant energy) are very effective but slightly less so than those between 400 and 500 nm; wavelengths

around 340 nm are much less effective than are wavelengths around 370 nm, suggesting a minimum sensitivity in the lower end of the long ultraviolet range; the wavelength of 315 nm is highly effective, more so than those in the visible range; and 254 nm is very highly effective. Although short wavelength UV (254 nm) is 1000 times more efficient than visible light, the mutagenesis-lethality ratio is higher for visible light than for short UV. Although lethality studies are incomplete at this time, we have measured lethality and filament formation under various experimental conditions. Under continuous visible light illumination, the percentage of chemostat cells that do not form microcolonies in the period of time in which most cells have divided 5 times, never exceeded 10% of the population, a value not more than double the nonviable cells in unirradiated cultures. Continuous irradiances of 10,000 ergs/mm²/sec, 10 times the highest intensity used in mutation studies, did not produce a significant reduction in cell titer nor an increase in the fraction of nonviable cells. Although chemostat cultures were subjected to routine examination, filaments were not observed in cultures irradiated with visible light. However, with long UV (355 ± 20 nm) irradiances of 200 ergs/mm²/sec or greater, cultures of filaments were produced. The filaments at these irradiances could be eliminated by the addition of pantoyl lactone. Irradiances of 1–2 ergs/mm²/sec at 254 nm also produced filamentation.

Anoxic results also are shown in Table 43. The mutation rate under anoxic conditions (95% N₂, 5% CO₂) for chemostat cultures irradiated with white fluorescent light (with a 424 nm sharp-cut filter) at an irradiance of 570 ergs/mm²/sec was 3.1 (S.E. ± 0.5) mutants per 10⁹ cells per hour, somewhat lower than the spontaneous mutation rate under anoxia. With long UV, at an irradiance of 110 ergs/mm²/sec under anoxic conditions, however, the mutation rate was 12 (S.E. ± 2) mutants per 10⁴ cells per hour, a value 2 or 3 times greater than the dark anoxic mutation rate and about one-fifth the rate obtained with black light at this intensity in the presence of oxygen. When a sharp-cut filter which removed wavelengths less than 340 nm was used with the long UV lamp, however, the anoxic response was not significantly higher than in the absence of the radiant energy. These results are consistent with a significant contribution from direct absorption by DNA (the anoxic response) for wavelengths below 350 nm.

Mutagenesis in the *E. coli* WP2-her⁻ is shown in Table 43. Mutation induction by unfiltered white fluorescent light was about three times more effective in this UV-sensitive strain than in *E. coli* B/r/1,try⁻; however, when wavelengths below 408 nm were filtered

FIG. 124.—Visible light and ultraviolet mutation rates (resistance to phage T5) in glucose-limited chemostat cultures. Growth rates were 0.18 to 0.22 division per hour.

FIG. 125.—Relationships between rate of mutation to resistance to phage T5 and wavelength of radiant energy in chemostat cultures. Growth rates were 0.18 to 0.22 division per hour.

out, the mutation rate fell to about one-fifth that of the other strain. Black light mutagenesis in this repair-deficient strain was 10 times more effective than in *E. coli* B/r/1,try⁻. These data are consistent with the view that *E. coli* WP2-her⁻, being 20 to 30 times more sensitive to effects by direct absorption of DNA, shows a high level of long UV mutagenesis from the direct absorption by DNA. Direct absorption is a much smaller component of long UV mutagenesis in the repairable strain *E. coli* B/r/1,try⁻.

Mutation to T5 resistance can be induced under conditions of free growth conditions in a turbidostat, a continuous culture without extrinsic control of growth rate. The visible light mutation rate in *E. coli*

B/r/1,try⁻ is approximately the same in chemostat and turbidostat cultures.

Preliminary results demonstrate that mutation to streptomycin resistance also can be induced with visible light. Thus, it is evident that visible light mutagenesis is not limited to a single strain of bacteria, nor is it limited to the induction of resistance to phage T5. Visible light mutagenesis may be a general phenomenon, dependent primarily on the presence of oxygen and a sufficiently high concentration of the chromophore(s).

CONCLUSIONS

The oxygen requirement for visible light mutagenesis is consistent with a photodynamic mechanism of action, but no conclusions are possible at present because the amount of the chromophore(s) present may be much smaller under anoxic conditions.

The nature of the chromophore(s) is of special interest because of its (their) possible association with the DNA of the cell. Several components of *E. coli* are possible candidates for the chromophore. Riboflavin, a planar three-ring molecule similar to the acridines, conceivably could bind to DNA through intercalation as Lerman has suggested for other such planar molecules.⁽⁶⁾ Riboflavin is photodynamically active in certain systems *in vitro*.⁽⁷⁾ Vitamin K and

any of several porphyrines, however, also are possibilities for the chromophore.

We postulate that the chromophore(s) for visible light mutagenesis may function in normal metabolic processes involving DNA. This relationship may involve intercalation of these planar molecules in DNA as a fundamental process in replication and transcription.

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GENETIC ANALYSIS OF A LARGE CELL STRAIN OF ESCHERICHIA COLI

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PURPOSE AND METHODS

A genetic analysis of *Escherichia coli* P6, a camphor-induced mutant of *E. coli* 82/r, is currently in progress in this Division. *E. coli* P6 has larger cells which contain about three times more DNA, RNA, and protein, and which are more resistant to radiation than those

TABLE 44. MACROMOLECULAR COMPOSITION AND CELL SIZE OF *E. coli* P6 AND 82/r

Culture	DNA/cell, g $\times 10^{-15}$	RNA/cell, g $\times 10^{-14}$	Protein/cell, g $\times 10^{-13}$	Median cell size, μ^3
Hfr P6	23.06 $\pm$ 1.14	7.39 $\pm$ 1.81	5.87 $\pm$ 0.71	1.27
P6-type re-combinants	28.05 $\pm$ 3.19	11.68 $\pm$ 0.75	6.68 $\pm$ 0.32	1.10
82/r F ⁻	7.95 $\pm$ 1.90	3.75 $\pm$ 0.53	1.70 $\pm$ 0.14	0.47
82/r-type re-combinants	8.43 $\pm$ 0.37	3.01 $\pm$ 0.29	1.95 $\pm$ 0.18	0.48
Ratio of P6: 82/r	3.23	3.32	3.43	2.40

of its parent. This study represents the first step in an attempt to understand the relationships among the various characteristic properties of this strain to one another and to the genetics and mechanisms of radio-resistance in *E. coli* P6. The characterization of the recombinants from the original mating of *E. coli* Hfr P6 with *E. coli* 82/r F⁻ strains⁽¹⁾ was completed by determining the macromolecular composition of stationary cultures, using standard analytical methods. The corresponding cell-size distributions in these cultures were obtained with a Coulter counter-analyzing system. The procedures used to introduce auxotrophic markers into an F⁻ strain of *E. coli* P6 and the techniques used in the reciprocal matings of this strain with an *E. coli* B Hfr strain (made available by Dr. H. Boyer⁽²⁾) also were presented in the last report.⁽¹⁾

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Table 44 shows the macromolecular composition and average cell size of the two parent strains and of three recombinant strains of each type from the original

mating. (Each value represents the mean of two separate experiments.)

Some quantitative differences between the values for the parents and their corresponding recombinants can be seen in this table, especially with regard to the P6 strains. Nevertheless, both recombinant types closely resemble their parents, with no indication of an intermediate class. The same conclusion is indicated for the cell size measurements and for the X-ray survival curves which were reported previously.⁽¹⁾

A statistical analysis performed on the macromolecular composition data further supports this conclusion. Very highly significant differences ($p < 0.001$) were obtained between the DNA, RNA, and protein contents of the two strains. The only significant difference ($p \simeq 0.05$) among cultures of the same strain was in their RNA content which appeared to be due almost entirely to a low value for the Hfr P6 culture.

Hfr B2 (thr^- , met^- , str^S , T_6^S) was used as the donor and two P6 strains (F^- , arg^- , iso^- , ade^- , str^R , T_6^R) as the recipients in the reciprocal mating experiments. The direction of chromosome transfer of Hfr B2 is the same as that of Hfr P6-2 (counterclockwise), but the origin is some six minutes closer to the locus tentatively assigned to the P6 "gene." (*Ade* and *met* are the first markers transferred, as compared to *leu* for Hfr P6-2). All of the markers in the two P6 F^- strains are isoallelic, with the exception of *arg*. This marker

maps at *arg* E in one strain, and at the *arg* A, C, F, H cluster in the other strain.

When Hfr B2 was mated with each of the two recipients in parallel experiments, the *str* marker was transferred 42 min and the P6 marker 44 min after the initiation of conjugation. The same relative order and distance between the two markers was demonstrated in the original mating.⁽¹⁾ Due to the considerable distance between the chromosomal origins of all available Hfr B strains and the position of the P6 and *str* markers, their exact loci are still somewhat uncertain. An attempt is being made at present to obtain finer mapping data through the use of transduction studies.

CONCLUSION

The results reported above support the conclusion drawn previously⁽¹⁾ that a single mutant gene is responsible for the complex character of the P6 strain. Experiments now in progress should add further support to this conclusion.

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AN AUTOMATIC TIME-PROGRAMMED SAMPLE COLLECTOR

William J. Eisler, Jr.

PURPOSE AND METHODS

Automation of an experimental biological system can lead to complex problems when off-hour sample collecting is involved. This situation is clearly demonstrated by the performance of an electronically controlled continuous culture device,⁽¹⁾ where fractions of the effluent from a bacterial growth tube were collected at time intervals of less than 1 hr. These collections were repeated every 2 hr, and can, therefore, be very demanding on research personnel, even during scheduled working hours. These demands are usually too severe to allow effective off-hour attendance of the investigator.

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The device, designed, constructed, and described here, to manage intermediate or overflow sampling up to 72 hr (Friday afternoon to Monday morning) com-

pletely frees the experimenter from excess attendance during an experiment that demands continual sampling. Commercially available fraction collectors did not satisfy these requirements because they are much too large to fit an incubator and do not have overflow disposal systems. Space requirements are important since the detector assembly and the sample collector usually must operate in a temperature-controlled incubator with space restrictions. Figure 126 shows the sample collector, its electronic control and the detector housing, with the tube to be sampled located in an operating position. Six sample positions are employed, each with a capacity of 30 ml. A method was devised that combines the sample collector and the sample overflow into a single position. This was accomplished by intercepting the sample overflow by a funnel, operated by a solenoid, that diverts the overflow into a common external reservoir. Two adjustable timers control the length of cycle

FIG. 126.—An automatic time programmed sample collector

FIG. 127—Block diagram showing logic sequence

either to sample the cultures or to allow the sample to overflow. Sample vials are suspended in an insulated transport container filled either with water or anti-freeze. An external, refrigerated water bath pumps a coolant through a coil located inside the transport container. Cells collected from the culture in the tube are kept cool (5°C) and in a dormant state.

A gear rack attached to the bottom of the sample

transport, is driven by a Bodine motor* with an appropriate matching gear. Dynamic braking of the motor assures precise, preselected sample positioning of the transport. Three microswitches and actuators, located on the side of the sample transport and housing, control positioning of the sample, motor reversal, and the end of the collecting cycle. Front panel controls allow the random sample advance and also control reversal of the sample transport. Illuminated sample indicator lamps display the *active* sample number. This is particularly helpful when the sample changer is operated remotely in an isolated or enclosed chamber (e.g., an incubator). Figure 127 is a block diagram of the complete system.

CONCLUSIONS

This sample collector has performed satisfactorily and is reliable. It is apparent that other types of biological applications are possible. For example, chemostat growth studies, having a 6-day duration, required no specific attention during this period. Samples would be collected automatically for 1 hr at 11-hr intervals

* Bodine Motor Type KYC-22 RC, 15 rpm. Bodine Electric Co., Chicago.

and weekend operation would be continuous from Friday afternoon through Monday morning. Appropriate timers are available commercially that meet almost any sample collection and/or sample overflow schedule.

I would like to acknowledge the assistance of Mr.

William Cole of Central Shops for the machining and mechanical assembly of this apparatus.

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Developmental Biology

THE BIOCHEMISTRY OF AMPHIBIAN DEVELOPMENT

Richard E. Ecker and L. Dennis Smith

PURPOSE AND METHODS

We are continuing our investigation on the metabolic control of synthetic processes in early amphibian development. Earlier we demonstrated that significant protein synthesis occurs in either fertilized or artificially activated eggs of the frog *Rana pipiens*, and the rate of this synthesis can be measured.⁽¹⁾ We have also shown that protein synthesis in the egg is initiated by administration of pituitary hormones to the adult female; the rate of this synthesis reaches its peak level well before the point in time when the eggs can be fertilized or artificially activated.⁽²⁾ Finally, we have presented evidence suggesting that the nucleus does not play an *active* role in the control of this synthesis.⁽²⁾

To assess the possible *passive* role of the egg nucleus in the control of synthesis during this hormone-induced maturation period, it was necessary to develop techniques to induce, *in vitro*, maturation of eggs removed from their ovarian follicles. This report deals with these techniques and some of their applications.

The methods used were essentially those described in past reports.^(1, 2)

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Full-grown oocytes dissected from their ovarian follicles can be induced to undergo physiological maturation by exposure, *in vitro*, to the steroid hormones progesterone and deoxycorticosterone acetate (DOCA) or to homoplastic pituitary suspensions.

When atfolliculate oocytes were exposed to the hormone continuously for 48 hr, as little as 0.1 $\mu\text{g/ml}$ progesterone or 0.001 $\mu\text{g/ml}$ DOCA induced normal maturation (as evidenced by activation) in all oocytes tested. Pituitary suspensions were less effective, induc-

ing maturation in less than 50% of the eggs at the optimum dose of $\frac{1}{160}$ pituitary/ml. We also discovered that at doses of 1 $\mu\text{g/ml}$, progesterone can induce maturation in about 30% of eggs exposed to hormone for as little time as 5 min and that exposure for 60 min with this dose routinely gives complete maturation of all eggs. Control oocytes in solutions without hormone do not mature. Although the steroid hormones are more effective inducers of maturation in atfolliculate oocytes, when ovarian fragments are used the pituitary suspensions more effectively induce both maturation of the oocytes and ovulation, i.e., release of the oocytes from the ovarian follicle.

To determine whether or not the oocytes which have matured *in vitro* are capable of cleavage and subsequent development, an artificial insemination procedure was used. Oocytes that have not passed through the oviducts of the female lack jelly envelopes, and it has not been possible to fertilize jelly-free eggs. Therefore jellyless oocytes, which had been induced to mature *in vitro*, were vitally stained and transplanted into an ovulating "foster" female. Twenty-four hours later the foster female was stripped of as many eggs as possible and they were fertilized. The stained, donor eggs could be specifically identified and were observed. Of 307 oocytes so treated and recovered in seven experiments, 229 cleaved and, of these, 201 developed normally into tadpoles.

The *in vitro* maturation technique we have described now makes it possible to enucleate oocytes at early stages, prior to the breakdown of the germinal vesicle,⁽²⁾ and determine the effect on oocyte protein synthesis of extended periods without this large nucleus. Nucleated controls can be studied under the same conditions. These experiments have shown the following: 1) A small amount of amino acid is incor-

porated into protein in a folliculate, nucleated oocytes left 48 hr *in vitro* without hormone, but the rate is only about 5 to 10% of that in hormone-treated oocytes. 2) Enucleated, a folliculate oocytes, treated with hormone either before or immediately after enucleation, can, after 24 or 48 hr, synthesize protein as well as the nucleated controls.

CONCLUSION

In view of the observations indicating that the steroid hormones are more effective on a folliculate oocytes, while the pituitary suspensions work best on eggs still in their follicles, we have considered the possibility that the steroids act directly on the oocyte to induce maturation, while pituitary hormones act through the follicular tissue. Whatever mechanisms are involved, the technique we have described provides a simple, routine procedure to induce maturation in

a folliculate oocytes. We have been able to use the technique to study the role of the germinal vesicle in the control of protein synthesis, both during and following the maturation period. Our earlier work indicated a possible correlation between the time of germinal vesicle breakdown and the onset of protein synthesis during maturation.⁽²⁾ Results presented here, however, show that the contents of the germinal vesicle have no quantitative effect on protein synthesis during this period.

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COMPARATIVE EFFECTS OF NEUTRONS AND X RAYS ON DROSOPHILA MELANOGASTER OOCYTES AND OOGONIA

Richard C. Dickerman*

PURPOSE AND METHODS

The purpose of these experiments was to determine the relative biological effectiveness (RBE) of fast neutrons as compared with X rays upon germ cell stages in female *Drosophila melanogaster*. Recent work^(1, 2) has emphasized the importance of cell stage in relation to radiation damage. Because of the nature of oogenesis in *Drosophila*, three discrete cell stages can be readily irradiated and sampled; oogonia (premeiotic), immature oocytes, and mature oocytes.

The flies used in these experiments were from a stock of *Drosophila melanogaster* Stephenville isogenic for the second and third chromosomes. From this isogenic stock a lethal free X-chromosome strain was selected and used in subsequent experiments.

King, Rubinson, and Smith⁽³⁾ described oogenesis in *Drosophila melanogaster* and divided the process into 14 stages. Three stages of female germ cells were used in the following experiments: stage 14 oocytes, which are mature eggs, stage 7 oocytes, which are the most mature stage in newly emerged females, and the earliest stage of oogenesis, stem-line oogonia. Stage 14 oocytes and stage 7 oocytes were obtained and irradiated by techniques previously described.⁽⁴⁾ To

obtain oogonia, virgin females were irradiated when five days old, individually mated with two males, and transferred to new media every three days for two weeks. New males were added if needed. At the end of this two-week period, which was assumed to be sufficient time for the female to lay all eggs in post-stem-line oogonial stages at the time of irradiation, new males were added and egg samples were collected. However, it is possible that some cystoblasts were included in this sample.⁽⁵⁾

For the irradiations the females were placed in a Lucite cell and constrained in a single layer by nylon mesh. The Lucite cell was then placed in a polystyrene petri dish with moist Kleenex to maintain humidity during the irradiation period. The X-ray irradiations were carried out at 250 kV, 30 mA, with 0.25 mm Cu, plus 1.0 mm Al filtration, (half value layer of 1.1 mm Cu). The dose rate was 155 rads/min. A Victoreen-r meter was used to measure the X-ray dose, and a conversion factor of 0.96 was used to convert roentgens to rads.

The neutrons were obtained from the Janus reactor in the Division of Biological and Medical Research at Argonne National Laboratory. The samples were placed on the central axis of, and one meter from, the high flux of the reactor. The neutrons had a mean energy of 0.2 to 0.3 meV and the dose rate was 45

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rads/min. The gamma contribution averaged 8.7% of the total dose. Dosimetry for the Janus reactor was carried out by Frank Williamson and Donn Jordan. Two ionization chambers were used: one with a plastic wall and acetylene filling which responded to gamma rays and neutrons; the other with a magnesium wall and argon filling, which responded only to gamma rays. The doses were calculated in terms of the "standard man" based on first collision of neutrons with hydrogen. Since "standard man" is based on 10% hydrogen and *Drosophila* females contain only 9.1% hydrogen,⁽⁶⁾ a correction factor of 0.91 was used to convert measured rads to a value applicable to *Drosophila*.

The end points used in this study were egg-to-adult survival and the induction of sex-linked recessive lethals. An attempt was also made to measure the induction of second chromosome recessive lethals.

Egg-to-adult survival was measured by transferring the mated females to new vials daily, collecting and counting the first 24 eggs per female, and then counting the adults that emerged. Sex-linked recessive lethals were detected by the "Base" technique.⁽⁷⁾ To detect second chromosome lethals a *Cy/Pm* balanced lethal stock was used.⁽⁸⁾ No more than ten F_1 offspring were tested from a single irradiated female to avoid the possible occurrence of large clusters of sex-linked recessive or second chromosome recessive lethals in oögonial samples. Any apparent mutant was retested the next generation to insure that it was a valid mutant. All recessive lethals detected have been retained as stock cultures and will be tested to determine relative positions on the chromosomes.

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Table 45 gives data for egg-to-adult viability in stage 14 oocytes, based on two replicates. The data

TABLE 45. EGG-TO-ADULT VIABILITY IN *Drosophila melanogaster* STAGE 14 OOCYTES IRRADIATED WITH NEUTRONS OR X RAYS

Radiation	Dose, rads	Number of eggs	Percent hatch $\pm$ S.E.	Corrected percent hatch
Neutron	0	1845	79.0 $\pm$ 1.0	100.0
	89	2088	70.7 $\pm$ 1.0	91.2
	178	1447	64.8 $\pm$ 1.3	82.0
	267	1773	55.3 $\pm$ 1.2	71.7
	355	1366	60.5 $\pm$ 1.3	78.6
X ray	0	1897	76.1 $\pm$ 1.0	100.0
	96	1762	74.2 $\pm$ 1.0	97.9
	192	1673	69.8 $\pm$ 1.1	92.1
	288	2355	61.7 $\pm$ 1.0	81.4
	384	2615	57.1 $\pm$ 1.0	75.0

FIG. 128.—Survival curves for irradiated *Drosophila melanogaster* stage 14 oocytes. Triangles represent X-irradiated oocytes. Circles represent neutron-irradiated oocytes.

TABLE 46. EGG-TO-ADULT VIABILITY IN *Drosophila melanogaster* STAGE 7 OOCYTES IRRADIATED WITH NEUTRONS OR X RAYS

Radiation	Dose, rads	Number of eggs	Percent hatch $\pm$ S.E.	Corrected percent hatch
Neutron	0	10109	80.4 $\pm$ 0.4	100.0
	267	6833	73.6 $\pm$ 0.5	90.9
	533	5898	69.6 $\pm$ 0.6	85.1
	800	2800	61.8 $\pm$ 0.9	73.7
	1066	5824	56.0 $\pm$ 0.6	68.3
X ray	0	3586	78.7 $\pm$ 0.7	100.0
	960	3002	69.0 $\pm$ 0.8	88.5
	1920	1901	62.8 $\pm$ 1.1	81.9
	2880	2621	55.9 $\pm$ 1.0	70.4
	3840	1416	47.0 $\pm$ 1.3	58.1

FIG. 129.—Survival curves for irradiated *Drosophila melanogaster* stage 7 oocytes. Triangles represent X-irradiated oocytes. Circles represent neutron-irradiated oocytes.

are plotted graphically in Figure 128, and a comparison of the slopes of the two curves indicates an RBE of 1.57.

Table 46 contains the results for egg-to-adult via-

TABLE 47. EGG-TO-ADULT VIABILITY IN *Drosophila melanogaster* OOGONIA IRRADIATED WITH NEUTRONS OR X RAYS

Radiation	Dose, rads	Number of eggs	Percent hatch $\pm$ S.E.	Corrected percent hatch
Neutron	0	8432	76.8 $\pm$ 0.5	100.0
	267	7362	74.5 $\pm$ 0.5	95.9
	533	7100	73.0 $\pm$ 0.5	91.3
	800	6699	66.6 $\pm$ 0.6	87.5
	1066	5149	69.8 $\pm$ 0.6	90.0
X ray	0	2375	77.4 $\pm$ 0.9	100.0
	960	2433	72.8 $\pm$ 0.9	93.5
	1920	2069	74.2 $\pm$ 1.0	92.3
	2880	2121	72.5 $\pm$ 1.0	90.7
	3840	1328	69.8 $\pm$ 1.3	87.4

FIG. 130.—Survival curves for irradiated *Drosophila melanogaster* oogonia. Triangles represent X-irradiated oogonia. Circles represent neutron-irradiated oogonia.

bility in stage 7 oocytes. These data are composed of eight replicates for the neutrons and four replicates for the X rays. A comparison of these curves, represented graphically in Figure 129, produces an RBE of 2.88.

The data for egg-to-adult viability in oogonia, based upon eight replicates for the neutrons and four replicates for the X rays, are presented in Table 47. There is a possibility that the samples contained some cells which were cystoblasts when irradiated.⁽⁵⁾ Oogonial cells must undergo four synchronous mitotic divisions to become a cystoblast (stage 1 oogonia). If a stem-line oogonial cell is injured by radiation, it is unlikely that these four divisions will be successfully completed, and this selection against damaged cells in mitosis could account for the seeming radiation resistance of oogonial cells which demonstrate an RBE of 3.89 (see Figure 130). In cystoblast cells, which have undergone the four synchronous mitotic divisions, the oocyte chromosomes are in late first prophase and the chromatids are held together by the synaptonemal complex.⁽⁵⁾ Any chromosomes broken by irradiation

would be held in position and repair could be quite as effective in removing dominant lethal events as cell selection against severely damaged stem-line oogonial cells. Notel⁽⁹⁾ has calculated that there is only one oogonial cell per ovariole, while King⁽¹⁰⁾ has estimated there are no more than two oogonia per ovariole. In either case, a high dose of irradiation would have a great probability of killing the stem-line oogonial cell, or cells. There was some indication that at the higher dose levels, fewer eggs were being produced by each female. Cytological work must be done to see if germ cell stem line deaths would lead to a decrease in the total number of eggs produced.

Table 48 gives the data for induction of sex-linked

TABLE 48. SEX-LINKED RECESSIVE LETHALS INDUCED IN *Drosophila melanogaster* STAGE 14 OOCYTES IRRADIATED WITH NEUTRONS OR X RAYS

Radiation	Dose, rads	Number of chromosomes	Lethals	Percent lethal	Percent induced lethals	Induced lethals	
						95% Fiducial limits	
						Lower	Upper
Neutrons	0	308	4	1.30	0.0		
	89	93	0	0.0	0.0		
	178	689	3	0.44	0.0		
	267	599	5	0.83	0.25	0.02	1.06
	355	303	5	1.65	1.28	0.35	3.37
X rays	0	308	4	1.30	0.0		
	96	795	8	1.01	0.0		
	192	771	6	0.78	0.0		
	288	200	2	1.00	0.0		
	384	382	4	1.05	0.76	0.16	2.29

TABLE 49. SEX-LINKED RECESSIVE LETHALS INDUCED IN *Drosophila melanogaster* STAGE 7 OOCYTES IRRADIATED WITH NEUTRONS OR X RAYS

Radiation	Dose, rads	Number of chromosomes	Lethals	Percent lethal	Percent induced lethals	Induced lethals	
						95% Fiducial limits	
						Lower	Upper
Neutrons	0	1995	23	1.15	0.0	0.00	0.18
	267	2277	21	0.92	0.33	0.12	0.63
	533	2312	31	1.34	0.60	0.32	1.01
	1066	1683	40	2.88	1.52	0.99	2.23
X ray	0	1089	5	0.46	0.0	0.00	0.33
	960	966	19	1.97	1.57	0.88	2.57
	1920	431	12	2.78	1.95	0.80	3.65
	2880	1197	36	3.01	2.61	1.78	3.68
	3840	508	24	4.72	4.37	2.78	6.55

recessive lethals in stage 14 oocytes. The number of induced lethals was calculated for each replicate experiment by determining the number of lethals expected in each sample, using the lethal frequency in the controls and subtracting this value from the number of lethals detected. At the lower level of irradiation the number of lethals recovered was less than, or equal to, the spontaneous mutation rate. Higher radiation doses could not be used with stage 14 oocytes because of dominant lethality induced in the eggs. A comparison of mutation rates based on the highest radiation dose indicates an RBE of 1.80.

The dose response for neutrons and X rays in the induction of sex-linked recessive lethals in stage 7 oocytes is very similar, indicating an RBE of 1.46 (see Table 49 and Figure 131).

The data for the induction of sex-linked recessive lethals in oogonia is presented in Table 50 and Figure 132. Some clusters of lethals were encountered in this

FIG. 131.—Sex-linked recessive lethals induced in *Drosophila melanogaster* stage 7 oocytes. Triangles represent X-irradiated oocytes. Circles represent neutron-irradiated oocytes.

TABLE 50. SEX-LINKED RECESSIVE LETHALS INDUCED IN *Drosophila melanogaster* OOGONIA IRRADIATED WITH NEUTRONS OR X RAYS

Radiation	Dose, rads	Number of chromosomes	Lethals	Percent lethal	Percent induced lethals	Induced lethals	
						95% Fiducial limits	
						Lower	Upper
Neutrons	0	1121	5	0.45	0.0	0.00	0.32
	267	1280	9	0.70	0.39	0.13	0.96
	533	1394	12	0.86	0.54	0.25	1.08
	1066	1064	12	1.12	0.89	0.42	1.66
X ray	0	431	4	0.93	0.00	0.00	0.85
	960	443	6	1.35	1.13	0.36	2.63
	1920	501	3	0.60	0.00	0.00	0.73
	2880	966	19	1.97	1.68	0.97	2.71
	3840	539	14	2.60	2.16	1.11	3.79

FIG. 132.—Sex-linked recessive lethals induced in *Drosophila melanogaster* oogonia. Triangles represent X-irradiated oogonia. Circles represent neutron-irradiated oogonia.

TABLE 51. MUTATION RATES IN NEUTRON AND X RAY IRRADIATED *Drosophila melanogaster* OOCYTES AND OOGONIA

	Neutrons	X rays	RBE
Dominant lethals			
Stage 14	$10.5 \times 10^{-4}/\text{rad}$	$6.67 \times 10^{-4}/\text{rad}$	1.57
Stage 7	$2.99 \times 10^{-4}/\text{rad}$	$1.04 \times 10^{-4}/\text{rad}$	2.88
Oogonia	$1.17 \times 10^{-4}/\text{rad}$	$0.30 \times 10^{-4}/\text{rad}$	3.89
Sex-linked recessive lethals			
Stage 14	$3.61 \times 10^{-5}/\text{rad}$	$1.98 \times 10^{-5}/\text{rad}$	1.80
Stage 7	$1.41 \times 10^{-5}/\text{rad}$	$0.97 \times 10^{-5}/\text{rad}$	1.46
Oogonia	$0.93 \times 10^{-5}/\text{rad}$	$0.53 \times 10^{-5}/\text{rad}$	1.74

stage. If two or more lethals occurred in the offspring from a single female, they were considered to be the result of a single mutational event, leading to a cluster of damaged eggs. Subsequent genetic mapping will be carried out to test the theory that all lethals in a cluster are identical. A comparison of the slopes of the two curves indicates an RBE of 1.74.

Tests were made to determine the frequency of induction of second chromosome recessive lethals using the *Cy/Pm* technique. Due to the large number of lethals occurring in the controls (116 lethals in 1198 chromosome scored) allelism tests will be made to determine the number of lethals actually induced in the irradiated groups. There were 581 lethals found in the 4511 irradiated second chromosomes tested.

CONCLUSION

Neutrons are more damaging than X rays, but this effect is more pronounced in dominant lethal events than in recessive lethal events. Data from both egg-to-adult survival tests and the sex-linked recessive lethal tests indicated oogonia are the least sensitive to irradiation, stage 14 oocytes the most sensitive, and stage 7 oocytes are intermediate. (See Table 51). At

stage 14 the eggs are relatively inactive metabolically, and Parker has indicated that breaks do not heal until the egg is reactivated by fertilization.⁽¹¹⁾ Stage 7 oocytes, on the other hand, are very active metabolically, and repair of broken chromosomes can take place in fifteen minutes or less.⁽¹¹⁾ Fractionation of doses of both X rays and neutrons to stage 7 oocytes indicated repair was possible following both types of irradiation. This result (repair following neutron irradiation) has not been reported previously and will be investigated further. It was postulated that the four synchronous divisions an oogonial cell must undergo to become a cystoblast (stage 1 oocyte) would serve as a strong selective pressure to eliminate grossly damaged (dominant lethal containing) oögonia.

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MECHANISMS OF CARCINOGENESIS

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PURPOSE AND METHODS

This is a continuing program whose central focus is investigation of the conditions under which tumors arise in locally irradiated tissues. It seems evident that a malignant tumor does not arise at once, even after a single, optimal exposure. The cellular dynamics in the irradiated epidermis are, accordingly, receiving special attention, in addition to other aspects of carcinogenesis discussed in previous reports^(1, 2) in which we described the methods employed here. This report will deal particularly with (1) production of sarcomas by implanted plastics, and the comparison of responses to radioactive and nonradioactive implants; (2) the growth patterns of benign mammary tumors in rats injected subcutaneously with plutonium-239; (3) conditions that may favor bone tumor induction by plutonium-239; (4) reactions of epidermal cells after beta irradiation in carcinogenic amounts. Quantitation of the cellular reactions has been accomplished by the measurement of epidermal thickness; measurements of nuclei and cells in the basal and upper layers; the enumeration of total, ³H-thymidine-labeled, and dividing cells, according to location; and the estimation of denudation and regrowth of epithelium.

PROGRESS REPORT

Low Dose Rate Irradiation of Mouse Epidermis

Survivors of external beta exposure (⁹⁰Sr-⁹⁰Y), at surface dose rates about 7.5 rads/min to totals of 5000 and 7200 rads (11 and 16 hours' exposure), have begun to develop malignant tumors at about a year after exposure. Larger numbers of mice are now being exposed and will be kept in order to measure the rates of tumor development. From various reports of experiments in which ⁹⁰Sr-⁹⁰Y exposures were given at higher dose rates (generally about 100 rads/min), it would appear that there is a rather large dose-rate factor with respect to cell death as reflected in denudation of the skin, since at the lower dose rate, ulceration has occurred only at the 7200-rad level. We plan to compare initial cellular effects with the tumor responses at different dose rates.

Cellular Responses in Irradiated Epidermis

Preliminary observations on hairless mice (5000 or 7200 rads at 7.5 rads/min) have shown the following: (1) DNA synthesis and cell division are suppressed for about 2 days; ³H-thymidine-labeling begins to exceed control levels during the third or fourth day and reaches a maximum (about 45% of basal cells) 7 days after 5000 rads and 10–11 days after 7200 rads; increased mitotic counts follow a qualitatively similar course, reaching levels of 1.5 and 2.5% of basal cells. (2) Hyperplasia, as indicated by an increase in ³H-thymidine-labeling, continues between 2 weeks and 3 months at a level of 10 to 12.5% of basal cells after 7200 rads and 4 to 8% after 5000 rads (control values at the same time of day are from 2 to 3%). (3) Mean nuclear volumes of the irradiated cells increase from the second or third day to a maximum at about one week; at that time, the mean volumes are about 7 to 10 times control, and some nuclei are much larger. These cells migrate to the surface and are lost, being replaced from below by cells of normal size. In areas where replacement does not occur (7200 rads), ulceration appears. (4) The total thickness of the epidermis, and of the overlying keratin, increases during the period of hyperplasia; the epidermis is consistently thicker than in controls of like age, as long as 4 months after irradiation at both levels.

It is evident that most of the irradiated cells have undergone genetic death but that they enlarge and continue to be metabolically active for several days, as indicated by continued ³H-thymidine uptake, up to the point of sloughing. Thus it would appear that the tolerance of epidermis to radiation may depend on the protective effect of the differentiating "killed" cells which fail to inhibit proliferation, as normal skin does.

Sarcoma Production by Subcutaneous Implants

A wide variety of plastic, metal, and other solid disks have been shown to produce sarcoma, when implanted subcutaneously. It has been reported that many needle-sized holes in the disks, or open-mesh disks prevented the production of tumors. Our experience with 0.3- μ pore Millipore disks, compared to 10- μ pore disks,

TABLE 52. SARCOMA PRODUCTION BY SUBCUTANEOUS IMPLANTS

Group ^(a)	No. of animals	Total No. of tumors	No. of animals with tumors	Percent of animals with tumor	Type of implant
A ₁	48	46	33	68.7	Teflon 27 mm, 2-mm holes on perimeter
A ₂	21	14	12	57.1	Teflon 27 mm, solid
A ₃	21	6	5	23.8	Teflon 17 mm, solid
A ₄	48	6	6	12.5	Teflon 27 mm, 9- μ pores
B ₁	50	26	23	46.0	Silastic 25 mm, 2-mm holes on perimeter
B ₂	25	12	10	40.0	Silastic 25 mm, solid
B ₃	25	8	8	32.0	Silastic 15 mm, solid
B ₄	50	26	23	46.0	Silastic 15 mm, perforated with 1-mm holes
C ₁	50	54	37	74.0	Cellophane 22 mm, 2-mm holes on perimeter
C ₂	25	25	18	72.0	Cellophane 22 mm, solid
C ₃	23	20	13	56.5	Cellophane 16 mm, solid
C ₄	50	44	31	62.0	Cellophane 16 mm, perforated with 1-mm holes
D ₁	48	72	44	91.7	Millipore 25 mm, 0.10- μ pores
D ₂	48	35	24	50.0	Millipore 25 mm, 0.22- μ pores
D ₃	41	5	5	12.2	Millipore 25 mm, 0.45- μ pores
D ₄	48	0	0	—	Millipore 25 mm, 1.2 μ pores
D ₅	50	0	0	—	Millipore 25 mm, 3.0- μ pores
D ₆	50	0	0	—	Millipore 25 mm, 8.0- μ pores
E ₁	50	14	11	22.0	Fiberglass 19 mm, 2- μ pores
F ₁	50	2	2	4.0	Reactor graphite 15 mm, solid

^(a) Groups A₁ to D₃ have been 600 days in experiment; Groups D₄ to F₁ have been 440 days in experiment. Each animal has 6 implants.

showed that a high incidence of tumors occurred with the 0.3- μ disks and very few with the 10- μ pore disks. In the case of the 10- μ pore disks, the capsule cells completely infiltrated the disks and very few tumors resulted. ⁽²⁾ More recent experiments have been designed to study the relationship of physical size, shape, and configuration of the implanted material to the tu-

mor incidence. Table 52 shows the present tumor incidences (600 days post-embedding) resulting from various implants. It appears that: (a) in the range of pore sizes from 0.1- μ to 0.45- μ Millipore disks, the incidence of tumors is inversely proportional to pore size. No tumors (440 days post-embedding) have yet been produced by Millipore disks with pore sizes from 1.2 μ to 8.0 μ , although 50% of the tumors in the small-pore groups arose before 500 days. (b) Disks of Silastic and cellophane with 20 one-mm holes punched in them produce as many tumors as solid disks of the same size. (c) Within the limits of size difference of disks tested (twofold area difference), the larger disks produce a higher incidence of tumors. (d) No difference in tumor incidence is seen, whether the disk is bound firmly by perforations at the perimeter or remains free in the capsule. (e) Two tumors have arisen 440 days after implanting reactor graphite disks. (f) The fiberglass prefilters are completely infiltrated by cells and show intense hyperplastic and giant cell activity; at 440 days post-embedding, the tumor incidence is substantial and is still increasing.

The tumors produced by these implants are all histologically diagnosed as sarcomas. The majority are fibrosarcomas, with lesser numbers classified as anaplastic and undifferentiated sarcomas, giant cell sarcomas and osteogenic sarcomas. Almost all the giant cell sarcomas and osteogenic sarcomas have been found in relation to cellophane implants. The different types of sarcomas produced are shown in Figure 133.

In Table 52, we have noted the percent of animals with one or more tumors. Each animal in the experiment received 6 subcutaneous implants of the same type. As a tumor developed, it was surgically removed, and the animal was kept in the experiment for the production of subsequent tumors. Up to four tumors have been removed from an animal before sacrifice was necessary due to recurrence of an inoperable tumor. The individual-disk probability of tumor formation is derived from the binomial expansion $(p + q)^6$. The percent of animals with no tumors is equal to the value of q^6 , and the percent of animals with one or more tumors equals $1 - q^6$. The frequencies of 1, 2, 3, 4, 5, and 6 tumors are given by the individual expansion terms. Table 53 shows the expected frequencies of animals with no tumors, and one or more tumors based on probabilities of individual-disk tumor formation ranging from 10 to 35%.

The percent of animals with one or more tumors associated with Millipore filters from Table 52 is 91.7%; Table 53 indicates that the individual-disk probability for this material is about 35% per disk.

The binomial expansion is applicable in situations involving independent events. Comparison of the

FIBROSARCOMA

FIBROSARCOMA
(LESS DIFFERENTIATED)

FIBROSARCOMA
(UNDIFFERENTIATED)

UNDIFFERENTIATED
SARCOMA WITH
GIANT CELLS

UNDIFFERENTIATED
SARCOMA WITH
MULTI-NUCLEATE
GIANT CELLS

OSTEOSARCOMA

FIG. 133.—Histologic types of sarcomas induced by implants

TABLE 53. TUMOR INCIDENCE EXPECTED FROM INDIVIDUAL DISK PROBABILITIES

Individual disk probability, %	Animals with 1 or more tumors, %	Animals with no tumors, %
10	46.9	53.1
15	62.3	37.7
20	73.8	26.2
25	82.2	17.8
30	88.3	11.7
35	92.5	7.5

FIG. 134.—Growth curves of benign breast tumors in ^{239}Pu -treated rats.

separate multiple tumor frequencies observed with the calculated frequencies (1–6 tumors), in the case of the 0.1- μ pore Millipore disks, demonstrates a good fit. This would indicate that the occurrence of each tumor is an independent event, with no relationship to the previous tumor production by other disks in the animal.

Implantations of Mylar-laminated ^{90}Sr - ^{90}Y , beta

FIG. 135.—Growth curve of benign breast tumor in untreated rat.

sources subcutaneously, containing 5 μCi , 2.5 μCi , and 0.5 μCi of ^{90}Sr - ^{90}Y , are producing capsule sarcomas more rapidly, and in a higher incidence, than nonradioactive Mylar implants. The present tumor incidence and the days to first appearance of tumor (365 days post-embedding) appear to be related to the amount of activity in the sources. The earliest tumor so far discovered appeared, and was identified both grossly and histologically, at 153 days after the implantation of a 5- μCi Mylar source.

The plastic disks and the ^{90}Sr - ^{90}Y Mylar sources implanted in 5 dogs have been in place for from 12 to 18 months. No tumors have, as yet, been observed.

Benign Breast Tumor Growth Rates

We have been measuring the growth rates of benign breast tumors, arising in Sprague-Dawley female rats injected subcutaneously with 8 injections of particulate ^{239}Pu . The benign tumors arising in these animals are of the same histological types (fibroadenoma,

adenofibroma, adenoma) as the senile benign breast tumors of the control rats. The majority of benign tumors in plutonium-injected rats, however, arise earlier than in the control series. The percent of animals with tumors was approximately the same in all of three different injected dose groups used, which may be attributed to the markedly decreased survival after higher dosages, since the average age to death was inversely related to the injected dose. The relation of dose to tumor production is shown more clearly by the incidence of multiple tumors. Twenty-five percent of the tumor-bearing rats, at the highest dose, had three or more tumors; this decreased to 9% at the intermediate dose and 6% at the lowest dose.

The growth rates, based on calculated volume of these benign tumors, were fitted by an exponential. Figure 134 shows the fit obtained for several ^{239}Pu benign tumors and Figure 135 shows a typical control benign tumor. The doubling times of the four tumors in Figure 134 range from 10 to 18 days, whereas the doubling time of the control tumor in Figure 135 is 35 days. The ^{239}Pu tumors typically had growth rates higher than control tumors. In addition, the highest dose of ^{239}Pu produced benign tumors whose mean and maximum growth rates were higher than those result-

FIG. 136.—Composite of tumor measurements in high-dose ^{239}Pu -treated rats.

FIG. 137.—Composite of tumor measurements in low-dose ^{239}Pu -treated rats.

ing from lower doses. This is shown in Figure 136 (56 tumors) and Figure 137 (41 tumors), in which the individual measurements of the tumors are plotted with time taken as zero when estimated tumor weight was one gram.

Plutonium-Induced Osteogenic Sarcoma

It was previously reported⁽²⁾ that castrate Sprague-Dawley female rats have a much higher incidence of osteogenic sarcomas than control animals, when injected subcutaneously with a similar dose of particulate ^{239}Pu . Studies are continuing to determine whether this difference is due to the production of a higher bone burden of ^{239}Pu in castrate rats. Alternative possibilities may relate to the longer period of rapid bone growth in castrate rats.

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RETENTION OF RADIOSTRONTIUM IN SOFT TISSUES

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PURPOSE AND METHODS

Our interest was originally drawn to this problem because of the possibility that genetic effects of ^{90}Sr - ^{90}Y in mice might be due to isotope deposited (and perhaps retained) in soft tissues, specifically in combination with gonadal DNA. From estimates of radiation dosage to the gonads of mice injected with ^{90}Sr - ^{90}Y made by fluorescing glass dosimeters, and from analyses of blood and tissues of mice injected with isotopes of strontium, we concluded that the mouse gonads receive substantial amounts of protracted radiation from nearby skeletal deposits but only transitory small doses from soft tissues including the gonads themselves.^(1, 2) From time-series of tissue and blood analyses, it was estimated that the total testicular integrated concentration is about 0.2 days (i.e., 0.2 μCi days/g tissue, per μCi /g body weight injected). The corresponding value for blood was about twice that for testes and for most of the soft tissues.⁽³⁾

As has been suggested before, it is quite possible that blood and soft tissues of larger animals might show a considerably greater retention of Sr, since it has long been known that most metabolic constants do not vary directly with body weight,^(4, 5) but according to some power of weight less than one. Comparison of reported plasma levels of ^{90}Sr after injection in the dog⁽⁶⁾ with our own observations on mice suggested a rather large factor of difference, and in order to check this we have followed plasma ^{85}Sr levels in a 12.5-kg beagle in the same way as we had done in mice, and have also examined the scattered data on various species that exist in the literature.

Since our previous report⁽²⁾ an independent confirmation of our original dosimetric measurements on testes of mice injected with ^{90}Sr - ^{90}Y has appeared.⁽⁷⁾

PROGRESS REPORT

A more detailed summary of the data on blood and soft-tissue levels in mice injected with radiostrontium has been published.⁽³⁾

A 12.5-kg male beagle about 3 years of age was given a tracer injection of ^{85}Sr (about 10 μCi /kg); blood and plasma samples have been measured at intervals from 5½ min to 167 days; 24-hr urine collections have also been taken and analyzed daily for 3 weeks and at intervals thereafter. It is evident that all or virtually all of the blood ^{85}Sr is in plasma. Urine: plasma ratios averaged 6 to 7 and plasma clearance to urine about 2000 ml/day.

A comparison has been made of some available data on plasma specific activity of injected mice, dogs, and human subjects (Figure 138). Specific activity is here defined in dimensionless units as above, viz. μCi /g of plasma relative to μCi /g body weight injected. Although many workers have reported series of plasma values, in a majority of cases the data are reported in such a way that assumptions as to values of parameters (e.g., body weight, total plasma volume, plasma calcium, and in our own mice hematocrit) have to be made in order to calculate the specific activity of plasma. The data shown have been taken from the following sources: for mouse, our own data on seven-week mice, combining series injected with ^{85}Sr and with ^{90}Sr ; for dog, our present data on ^{85}Sr and data taken from Stover and Atherton;⁽⁶⁾ and for man, data from Marshall's analysis of a patient of Spencer⁽⁸⁾ and from the "normal" case in the series studied by Dow and Stanbury.⁽⁹⁾ It appears that the final power-function slopes are roughly the same for the three species, but there is a very large difference in the time of inception of these slopes: a specific activity value of 1 is passed in about 0.2 days in the mouse, 1 day in the dog, and 6 days in man. Published data on the weanling rabbit⁽¹⁰⁾ would indicate that this animal follows a course similar to that of the mouse.

FIG. 138.—Plasma specific activity values following injection of radiostrontium in mice, dog, and human subjects. See text for sources of data.

CONCLUSIONS

The data are hardly sufficient to permit any assumptions as to the mechanisms underlying these differences, but it is clear that there are large differences between rate constants which determine the earlier phases of loss from plasma. Marshall⁽⁸⁾ has discussed in detail the possible influence of the factors which are involved in calcium and strontium retention. The degree of the differences also suggests that it would be unwise to assume an equivalent difference in soft tissue levels without direct measurements, but it can probably be assumed that the tissue dosages from radiostrontium in mice and human subjects differ by one or two orders of magnitude.

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THE ESTROUS CYCLE AND DNA SYNTHESIS IN THE MAMMARY GLAND

Harold Sutton and Katherine Suhrbier

PURPOSE AND METHODS

Patterns of epithelial cell proliferation of the mammary gland are under study as part of a larger study of mammary gland carcinogenesis in the rat. Bulough⁽¹⁾ has shown by the study of mitotic indices that the pattern of breast epithelial cell proliferation could be related to the ovarian cycle of rodents. Using autoradiographic techniques, we are now defining more precisely the proliferative activity of the epithelial cell populations in relation to the specific phases of the estrous cycle. Cells double their DNA prior to cell division so that the uptake of labeled precursor, tritiated thymidine, in newly synthesized DNA, is a convenient measure of cell proliferation. We have previously shown that in rats given a pulse of tritiated thymidine, the specific activity of extracted breast DNA varies with each of the four phases of the estrous cycle.⁽²⁾ This determination has the advantage of sampling large portions of the gland and is a relatively rapid method in comparison to the autoradiographic techniques. The breast is, however, a mixture of cell populations, including mainly supportive connective tissue cells, fat cells, and epithelial cells. The DNA specific activity determination gives an average picture of the proliferative activity of all

the cell populations in the gland and may not closely indicate the proliferative activity of the epithelial cells.

Mammary tumors arise from epithelial cells.⁽³⁾ It is, therefore, important to learn more about the proliferative activity of these cells. Autoradiographic techniques offer a way to differentiate the proliferative activity of specific cell populations. In this study, the percent of ³H-thymidine-labeled cells is used as a first approximation of the proliferative activity. We have asked basically three questions: (1) Does the percent of thymidine-labeled cells of such subpopulations of mammary epithelial cells, as duct and alveolar epithelial cells, vary in relation to the phases of the estrous cycle? (2) If they vary, does this occur in a uniform or in a dissimilar way? (3) Does the total cell proliferation of the mammary gland, as measured by the specific activity of extracted DNA, closely reflect the proliferative activity of certain subpopulations, as measured by the percent of ³H-thymidine-labeled cells?

As in previously reported experiments,⁽²⁾ young adult female albino rats, supplied by Charles River and derived from the Sprague-Dawley strain, were divided into four groups according to phase of the estrous cycle. A total of twelve rats, three in each

TABLE 54. LABELING INDICES OF MAMMARY GLAND EPITHELIAL CELLS

Estrous cycle phase	DNA specific activity, dpm/ μ g DNA	Labeling index, cells labeled/100 cells counted	
		Duct	Alveolus
Proestrus	115.5	0.80	0.77
Estrus	134.8	1.73	4.03
Metestrus	177.0	4.46	8.30
Diestrus	71.2	0.41	0.26

group, were given a standard pulse dose of tritiated thymidine, and one hour later these animals were sacrificed. A small biopsy was obtained from the second gland of the posterior gland group, and the balance of the paired, posterior group of three mammary glands was processed in the usual manner for subsequent determination of specific activity of DNA. From autoradiographs prepared from the biopsy of each animal, the percent of labeled cells was determined from counts of approximately 2000 duct and 2000 alveolar cells. Terminal bud cells were not included in this analysis because of insufficient numbers. We recognized that the distinction between duct cells and alveolar cells is not always clear, but the incidence of this ambiguity does not appear to be sufficiently high to invalidate the conclusions drawn from this study.

PROGRESS REPORT

The average labeling indices (percent of ^3H -thymidine-labeled cells) of two groups of the mammary gland epithelial cell population fluctuate in a similar pattern in relation to the estrous cycle. These labeling index patterns mimic, in general, the pattern of fluctuation seen in the specific activity of extracted mammary gland labeled DNA. As is shown in Table 54 the peak cell labeling indices are found during metestrus for both the alveolar cells and the duct cells, contrasting with the low values recorded during diestrus. Although it is evident that the labeling indices of both subpopulations rise and fall during the same phase of the estrous cycle, the alveolar cell labeling index increases to twice the level reached by the duct epithelium during estrus and metestrus. Both subpopulations are in close agreement during proestrus and diestrus.

Maximum levels of DNA specific activity are recorded in metestrus, and minimum values occur in diestrus. The patterns of the labeling index and the DNA specific activity are similar. This similarity suggests that a marked rise or fall seen in the specific activity signifies that more epithelial cells are entering

or leaving DNA synthesis. As can be seen from the patterns presented by the data, however, there is no consistent directly proportional relationship between DNA specific activity and epithelial cell labeling index for each phase of the cycle. There may be several reasons for this inconsistency. First, it may be a matter of sampling. Since biopsy material yields the labeling indices from a single area of the gland, it may be less representative of the total gland than the aliquot of the gland used for specific activity determinations.⁽²⁾ Second, the possible fluctuation of other cell populations in the mammary gland influencing the DNA specific activity may be independent of the epithelial cell pattern of fluctuation. Third, the DNA specific activity may be altered by a change of the rate at which individual cells synthesize DNA. Bresciani has offered evidence that mouse mammary alveolar epithelial cells respond to ovarian hormone changes by a twofold variation in the DNA synthesis time.⁽⁴⁾ But the data in the present study suggest that in spite of the role these factors may play, there is a qualitative relationship between mammary gland DNA specific activity and the ^3H -thymidine-labeling index of epithelial cells.

CONCLUSION

The proliferative activity of two subpopulations of mammary gland epithelial cells were defined during the phases of the estrous cycle by measuring the percent of ^3H -thymidine-labeled cells from autoradiographs. Both duct cells and alveolar cells fluctuate in a similar pattern in relation to specific phases of the estrous cycle. These cell subpopulations show maximum labeling indices during metestrus and minimum values during diestrus, but each fluctuates to a different degree. In cycling adult female rats, the specific activity of the extracted pulse-labeled mammary gland DNA fluctuates in a pattern related to the four phases of the estrous cycle and is an indicator of epithelial cell proliferation.

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ULTRASTRUCTURE OF RAT MAMMARY GLAND DURING THE ESTROUS CYCLE

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PURPOSE AND METHODS

Interest in the ultrastructure of the mammary gland, in this laboratory, stems from questions regarding the process of carcinogenesis in this organ. The gland is potentially involved in the physiological processes of proliferation, synthesis, involution, and lactation, which influence ultrastructure.^(1, 2) A more complete understanding of the ultrastructural changes taking place during these normal functions is necessary. This may aid in the identification and understanding of significant changes leading to the malignant process. Mammary cancer is readily induced in certain strains of albino rats exposed to whole-body X ray and chemical carcinogens.^(3, 4) This process occurs in a gland in which cell proliferative activity varies as a function of the estrus cycle.^(5, 6) Therefore, the ultrastructural appearance of the mammary gland of the untreated female rat is under study during the various phases of the estrous cycle.

Sixty-day-old virginal Charles River SD rats are vaginally smeared, and rats in proestrus, estrus, metestrus, and diestrus are selected for sacrifice. The inguinal mammary fat pad containing the mammary gland is removed and the tissue is initially fixed in cold glutaraldehyde. Using a dissecting scope, it is possible to identify ducts and alveolar buds after the tissue is stained with a 2% saturated thionin solution. Samples of these structures are removed and secondarily fixed in OsO₄. They are embedded in araldite-Epon media and sectioned with a Huxley microtome. After staining with uranyl acetate and lead citrate, the sections are studied in a Siemens Elmiskop I electron microscope.

PROGRESS REPORT

A limited number of mammary alveolar buds from rats in each of the four phases of the estrous cycle have been studied with the electron microscope. Distinct differences are seen in the ultrastructure of the alveolar buds as a function of the estrous cycle. To illustrate some differences, micrographs of cells in mammary alveolar buds of rats in estrus and diestrus are shown.

In estrus (Figure 139), many of the epithelial cells contain large fat droplets. Closely associated with the

fat droplets are often the larger mitochondria of the cell. The delimiting membrane of the mitochondrion, which borders the fat droplet, is often indistinct, or apparently missing. Organized rough endoplasmic reticulum (ER) is readily seen.

By contrast, in diestrus (Figure 140), the epithelial cells of the alveolar buds contain shorter and more scattered rough endoplasmic reticulum. Free ribosomes are very prominent in this stage. Fat droplets are greatly reduced in number. It may be of metabolic significance that the matrix granules, so conspicuous in the mitochondria of the diestrus mammary gland alveolar-bud cells, are less common in the mitochondria of the estrus cell.

CONCLUSION

1. Alveolar buds from mammary glands of cycling female rats can be selectively obtained and processed for study with the electron microscope.

2. There are alterations in the ultrastructure of the mammary gland alveolar buds as a function of the estrus cycle in the albino rat. These differences should be further defined and their significance explored.

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FIG. 139.—A view of several cells of alveolar buds from mammary gland of estrus rat. M_1 , typical mitochondrion; M_2 , mitochondrion adjacent to fat droplets; F, fat droplet; ER, rough endoplasmic reticulum.

FIG. 140.—A view of several cells of alveolar buds from mammary gland of diestrus rat. ER, rough endoplasmic reticulum; FR, free ribosomes; MG, matrix granule.

CARCINOGENESIS AND CELLULAR PROLIFERATION

Allan B. Reiskin and Anthony R. Sallee

PURPOSE AND METHODS

This project is designed to determine whether or not the rate of cellular proliferation is related to carcinogenic processes and reflected in tissue susceptibility, latent period, or tumor growth rate. There are experimental data which suggest that DNA synthesis is required for neoplastic transformation⁽¹⁾ and that tissue reactivity to treatment with carcinogens is related to the relative distribution of cells in the different phases of the generation cycle.⁽²⁾

Kinetic parameters are being measured in hamster cheek pouch epithelium with conventional autoradiographic techniques, first, to define changes which may occur as a consequence of aging, and second, to characterize the proliferative patterns which prevail under stress or during healing processes. A variety of carcinogenic agents will then be used to induce neoplastic transformation in cheek pouch epithelium with different kinetic patterns.

Previous experiments demonstrated kinetic differences in the pouch epithelium of newborn (6- to 12-hour-old) and adult (2- to 4-month-old) hamsters.⁽³⁾ However, technical difficulties associated with protracted experiments on newborn animals complicate their use as test objects for assessing carcinogenic responses. Therefore, these experiments are being extended to examine the kinetics of intermediate age groups.

PROGRESS REPORT

Male Syrian hamsters, 26 days old, from two inbred (DEA/ANL and PD4/ANL) and two random-bred (Crm/ANL and Gd/ANL) strains were injected intraperitoneally with tritiated thymidine (³HTdR, 0.5 μ Ci/g, specific activity 0.36 Ci/mM) and killed at varying intervals thereafter. Plots of the percent of mitoses labeled by ³HTdR in cheek pouch basal epithelium as a function of time in several age groups are shown in Figure 141. Data points have been omitted from the graph because (1) the neonatal and adult curves were published previously,⁽³⁾ (2) some of the data are preliminary, (3) it is difficult to assign meaningful confidence limits to individual points, and (4) there is no overlap of data points on the descending limbs of the curves. The curves do not cover a full generation cycle, but the area under the wave of labeled mitoses is a direct measure of the mean duration of DNA synthesis, and the remaining phases of the cycle can be computed according to Wright's hypothesis.^(4, 5)

The duration of DNA synthesis is clearly shorter in the cheek pouch epithelium of newborn and 26-day-old hamsters than in adults. The computed cell cycles for cheek pouch epithelium in adult, 26-day-old, and newborn hamsters are 155, 95, and 29 hr, respectively. There was some interest in relating proliferative growth in the cheek pouch to growth in other hamster tissues, and autoradiographs of jejunum were also prepared. Figure 142 shows the percent of jejunal mitoses labeled by ³HTdR as a function of time in 26-day-old hamsters. The mean duration of DNA synthesis is approximately 6 hr and the mean generation time is 11 hr. Although we do not have comparable data on adult hamsters, these figures, in the perspective of other studies on intestinal proliferation in rodents, suggest that the cell cycle is comparatively short, and may reflect developmental growth.

In addition to measuring proliferative changes associated with aging, other experiments are in progress to assess the feasibility of using wounding or environmental stress as methods for altering cheek pouch kinetics.

Several hamsters were placed in a darkened cold room (5°C) in the hope of inducing hibernation. When no signs of torpor developed within 48 hr, the animals were injected with ³HTdR and sacrificed within an hour. Preliminary evaluation of autoradiographs indicated that both the percent of cells labeled by ³HTdR and the mitotic index were within normal values.

In another series of hamsters a study was made of the effect of autografting on cell proliferation of pouch epithelium. Preliminary comparisons of autoradio-

FIG. 141.—The percent of mitoses labeled by ³HTdR in cheek pouch basal epithelium of 6- to 12-hour, 26-day and 8- to 12-week-old hamsters as a function of time after injection.

FIG. 142.—The percent of mitoses labeled by $^3\text{HTdR}$ as a function of time after injection in jejunal epithelium of 26-day-old hamsters.

graphs of $^3\text{HTdR}$ labeled autografts and of cheek pouch growing *in situ* have revealed that while the grafts are not completely established, the percent of

labeled cells after a pulse of $^3\text{HTdR}$ is 1.5 to 2.0 times greater than in the undisturbed tissue. However, toward the second postgrafting month the labeling patterns of undisturbed and grafted pouch tissue are indistinguishable on the basis of present data. Similar experiments with homografts are in progress.

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ENZYME REGULATION IN RAT LIVER

Carl Peraino

PURPOSE AND METHODS

As a continuation of previously reported studies on enzyme regulation,^(1, 2) the effects of several different glucocorticoids on enzyme regulation are being compared; and in addition the effects of other hormones (e.g., thyroxine and insulin) on the action of glucocorticoids is being studied. SD/ANL [ANL/66] rats are fed a protein-free diet for 5 days, after which their diet is changed to one containing 60% protein. Hormonal treatment is begun on the day of the dietary change and is continued throughout the experiment (7 days). Glucocorticoids, such as cortisone, prednisone, hydrocortisone, and triamcinolone, are given in single daily intramuscular injections at 6 different dose levels (5/1, 5/2, 5/4, 5/8, 5/16, 5/32 mg per dose); thyroxine or insulin is similarly administered at levels of 10 μg and 4 IU respectively. Five rats from each group are killed each day, and their livers are analyzed for four adaptive enzymes (serine dehydratase, ornithine transaminase, glucose-6-phosphate dehydrogenase, and glucokinase). This experimental approach provides information on:

- (1) The individuality of the response pattern of each enzyme to the administration of a single hormone

- (2) The relationship of the structure of glucocorticoids to their role in enzyme regulation
- (3) The effect of hormone interactions on enzyme regulation.

PROGRESS REPORT

The data for serine dehydratase and glucokinase at the 5/1- and 5/2-mg dose levels of cortisone and prednisone are given in Figure 143. At the 5/1-mg dose level, prednisone is more effective than cortisone in increasing serine dehydratase; but this difference in effectiveness is not apparent at the 5/2-mg dose level. The response of serine dehydratase to both steroids was reduced when the dosage was decreased from 5/1 to 5/2 mg. Glucokinase, however, was not reduced but was, instead, increased after the decrease in steroid dosage. At the 5/2-mg dose level, cortisone produced a consistently greater response in glucokinase than did prednisone, whereas at the 5/1-mg dose level the relative effectiveness of the steroids changed as the experiment progressed. These data represent a small part of the comprehensive picture obtained using six different dose levels of four steroids and studying the responses of the four different adaptive enzymes.

In preliminary studies on the effect of thyroxine on

FIG. 143A.

FIG. 143B.

FIG. 143C.

FIG. 143D.

FIG. 143.—The effects of cortisone and prednisone, given at two different dose levels, on the activities of serine dehydratase (A and B) and glucokinase (C and D) over a period of 7 days.

the action of cortisone, it was found that thyroxine treatment completely abolished the stimulatory effect of cortisone on serine dehydratase, but did not alter the inhibitory effect of cortisone on ornithine transaminase.

CONCLUSION

Results obtained to date indicate that the regulatory system for each of the four enzymes has a unique dose-time response pattern with respect to the action of cortisone, and that prednisone produces a somewhat different set of patterns. An indication has also been

obtained that additional treatment with thyroxine alters the regulatory effect of cortisone differently for each enzyme.

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METABOLIC AND MORPHOLOGIC STUDIES OF HEPATOCARCINOGENESIS IN THE RAT

Carl Peraino

PURPOSE AND METHODS

In the 1966 Annual Report⁽¹⁾ I described the initiation of a study of hepatocarcinogenesis in the rat, involving the feeding of the hepatocarcinogen, diethylnitrosamine. Study with this carcinogen was discontinued, owing to the hazards of working with it. The results obtained to this time indicated, however, that the experimental approach is valid, and a second study was initiated (3/2/67) with acetylaminofluorene (AAF) as the carcinogen. The effects of phenobarbital on the action of AAF were studied as they had been with diethylnitrosamine.⁽¹⁾

Four hundred, male weanling SD/Anl [Anl/66] rats were divided into eight groups. All groups received a 30% protein diet supplemented as follows: group 1, no addition (control); group 2, 0.05% phenobarbital; group 3, 0.005% AAF; group 4, 0.01% AAF; group 5, 0.02% AAF; group 6, 0.05% phenobarbital and 0.005% AAF; group 7, 0.05% phenobarbital and 0.01% AAF; group 8, 0.05% phenobarbital and 0.02% AAF. Three rats from each group were killed at intervals identified in Figure 144. Their livers were removed and portions were prepared for enzyme analysis and histological and ultrastructure examination by both light and electron microscopy. Histological examination and autoradiography were performed by Dr. R. J. M. Fry of this Division and the electron microscopy is under the direction of Dr. H. C. Pitot at McArdle Laboratory, University of Wisconsin.

PROGRESS REPORT

Figure 144 shows the change in the ratio of liver weight to body weight, and the tumor incidence in groups 1, 2, 5, and 8 throughout the experimental period.

FIG. 144.—Effects of AAF and phenobarbital, fed alone or in combination, on the ratios of liver weights to body weights in rats over a period of 175 days.

Rats fed 0.05% phenobarbital in the diet had a consistently higher liver weight-to-body weight ratio than the controls (30% protein alone). Rats fed 0.02% AAF in the diet showed an increase in the liver weight-to-body weight ratio beginning after day 42. This

change was accompanied by severe cirrhosis, and highly differentiated hepatomas began to appear by day 72. The livers increased progressively in size; the incidence of hepatomas also increased. All rats in this group examined after day 120 had multiple large hepatomas. Numbers below points on the curves indicate how many rats had hepatomas in each group of 3 rats killed at each interval. By day 175, all rats in group 5 also had mammary adenocarcinomas and inner ear tumors. Fourteen of the remaining 17 rats in this group had died within two weeks after day 175.

Before day 57, rats fed both 0.02% AAF and 0.05% phenobarbital in the diet had a higher liver weight-to-body weight ratio than rats fed only 0.02% AAF. Subsequently, however, this ratio was maintained at a lower level in rats receiving both phenobarbital and AAF than in rats receiving only AAF. Rats receiving both phenobarbital and AAF did not develop hepatomas until approximately 60 days after tumors appeared in rats receiving only AAF. In gross appearance, the livers of rats receiving both compounds had much less severe cirrhosis than was characteristic of the livers of rats on AAF alone. There were no mammary or inner ear tumors in rats that received both phenobarbital and AAF. Only 3 of the remaining 20 rats in this group died during the two weeks after day 175.

POSTNATAL GROWTH OF BIRDS AND MAMMALS

Anna Kane Laird

PURPOSE AND METHODS

In recent studies⁽¹⁻⁴⁾ we have shown that both the growth of embryos and their parts and the growth of tumors can be described over great ranges of size increase by an equation of the Gompertz type, based on the observation that in these widely different systems the specific growth rate tends to undergo an exponential decay with time.⁽⁵⁾

In the present study the postnatal growth of a variety of mammalian and avian organisms was fitted by the same mathematical model. However, for many species a linear growth process is also present after birth, which fits a straight line after the early, rapid growth process is completed. A compound mathematical model has been developed in which the instantaneous rate of the linear growth process is regarded as being proportional to the mass already attained by an underlying Gompertz growth process.

Space limitations preclude a description of the data obtained on the four enzymes studied in this experiment (serine dehydratase, ornithine transaminase, glucose-6-phosphate dehydrogenase, and glucokinase). Each of the enzymes was unique in its response to the treatments, but all showed progressive changes. The parts of the experiment involving the lower dosage levels of AAF are still in progress. Histological observations, including autoradiographs, are still being evaluated and specimens are still being processed for electron microscopy.

CONCLUSIONS

The results obtained thus far have shown that progressive changes occur in several properties of the liver during AAF carcinogenesis. It remains to be seen which of these changes are primarily related to hepatocarcinogenesis. The discovery of the ability of phenobarbital to antagonize the effects of AAF will greatly aid, in subsequent studies, in separating the toxic (cell killing) and neoplastic (cell transforming) effects of AAF.

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PROGRESS REPORT

The data analyzed were obtained from the compilation edited by Altman and Dittmer⁽⁶⁾ for the following species (table numbers in parentheses): cow (85), goat (86), swine (88), horse (89), cat (90), dog (91), guinea pig (92), hamster (93), mouse (94), rat (95), rabbit (96), chicken (97), turkey (98), quail (99), duck (99), and goose (99). Data for the shrew were obtained from Conoway.⁽⁷⁾

The Gompertz growth equation is the same as that used in previous studies of this series:

$$W_g = W_0 \text{Exp} \left[\frac{A_0}{\alpha} [1 - \text{Exp}(-\alpha t)] \right], \quad (1)$$

in which W_g is the weight at time t , W_0 is the initial weight for the period of study (the birth weight in the present case), and the A_0 and α are constants. A_0 is the

initial specific growth rate and α is the rate of exponential decay of the specific growth rate.

In order to fit the postnatal growth of many species of birds and mammals, we have extended this Gompertz growth equation to include a linear component of growth, which is regarded as being proportional in its rate at any instant to the body weight already achieved by the underlying Gompertz growth process. The weight of the animal at time t is then represented by the expression

$$W = W_G + W_L, \quad (2)$$

in which W_L is the weight accumulation from birth to time t due to the linear growth process, and

$$W_L = \beta \int_{t=0}^{t=t} \frac{W_G}{W_G(\infty)} dt. \quad (3)$$

Since $W_G = W_0 e^{A/\alpha} \cdot e^{-A/\alpha \cdot e^{-\alpha t}}$ and $W_G(\infty) = W_0 e^{A/\alpha}$,

$$W_L = \beta \int_{t=0}^{t=t} e^{-A/\alpha \cdot e^{-\alpha t}} dt. \quad (4)$$

Using the substitution $Z = A/\alpha \cdot e^{-\alpha t}$,

$$W_L = -\frac{\beta}{\alpha} \int_{Z=(A/\alpha)}^{Z=(A/\alpha)e^{-\alpha t}} \frac{e^{-Z}}{Z} dZ. \quad (5)$$

This expression was integrated by expanding e^{-Z} in a power series, which was summed to the point at which the next term would alter the sum by less than 0.1%. For each set of growth data, the values of W_0 , A_0 , α and β were estimated with the aid of a digital computer, using an iterative least squares procedure that in principle was similar to that used in our previous studies.

Considerable differences were observed among the growth constants of this sample of animals, as indicated in Table 55. The mammalian and avian species are each listed in this table in the order of increasing birth weight. When placed in this order, the mammals are likewise listed in order of decreasing values for α (with one exception), and also, with only few exceptions, in the order of decreasing values of A_0 , the initial specific growth rate. The avian species show no such regularity. Because the time scale of the growth process, measured as the interval between any two corresponding points on the growth curve, is inversely proportional to α ,⁽⁵⁾ the list of Table 55 indicates a strong tendency for the smaller mammals to pass through corresponding segments of the same growth curve in shorter intervals of calendar time than is required by the larger mammals. Table 55 also indicates that small mammals generally have a higher initial specific growth rate than do large mammals.

When the growth constants of the postnatal curves were compared with those of prenatal growth in the

TABLE 55. SPECIES DIFFERENCES IN GROWTH CONSTANTS

Species	Computed birth weight (W_0)	Initial specific growth rate (A_0)	Rate of decay of growth rate (α)
Mammals			
Shrew	0.35 $\pm$ 0.03 g ^(a)	0.361 $\pm$ 0.047 ^(a)	0.144 $\pm$ 0.025 ^(a)
Hamster	1.81 $\pm$ 0.08 g	0.225 $\pm$ 0.008	0.054 $\pm$ 0.002
Mouse	1.88 $\pm$ 0.17 g	0.115 $\pm$ 0.009	0.045 $\pm$ 0.003
Rat	5.60 $\pm$ 0.35 g	0.130 $\pm$ 0.004	0.033 $\pm$ 0.001
Rabbit	64.2 $\pm$ 4.9 g	0.119 $\pm$ 0.007	0.029 $\pm$ 0.002
Cat	93.9 $\pm$ 4.0 g	0.064 $\pm$ 0.004	0.022 $\pm$ 0.002
Guinea pig	102 $\pm$ 4.1 g	0.036 $\pm$ 0.002	0.018 $\pm$ 0.001
Dog	250 $\pm$ 7.9 g	0.066 $\pm$ 0.002	0.017 $\pm$ 0.001
Swine	1.68 $\pm$ 0.10 kg	0.079 $\pm$ 0.003	0.017 $\pm$ 0.003
Goat	3.50 $\pm$ 0.17 kg	0.026 $\pm$ 0.001	0.014 $\pm$ 0.001
Cow	36.3 $\pm$ 1.2 kg	0.011 $\pm$ 0.0004	0.0044 $\pm$ 0.0002
Horse	53.3 $\pm$ 3.1 kg	0.012 $\pm$ 0.001	0.0055 $\pm$ 0.0007
Birds			
Quail	4.16 $\pm$ 1.6 g	0.211 $\pm$ 0.020	0.071 $\pm$ 0.007
Chicken	31.2 $\pm$ 2.9 g	0.104 $\pm$ 0.004	0.026 $\pm$ 0.000
Goose	45.2 $\pm$ 3.6 g	0.283 $\pm$ 0.006	0.064 $\pm$ 0.002
Turkey	54.4 $\pm$ 18 g	0.096 $\pm$ 0.009	0.025 $\pm$ 0.000
Duck	56.3 $\pm$ 11 g	0.187 $\pm$ 0.027	0.042 $\pm$ 0.016

(a) Computed value $\pm$ standard error.

species for which we have previously analyzed the embryonic growth curve,⁽⁴⁾ we found that for most species the terminal specific growth rate of the prenatal growth period is not the same as that initiating postnatal growth, and furthermore, the rate of exponential decay of the specific growth rate also changes sharply at about the time of birth, usually being reduced to about 1/3 its previous value.

In Figure 145 several compound growth curves are shown, in which the weight and time scales have been normalized in terms of the underlying Gompertz curve.⁽⁵⁾ The early rapid growth of these organisms is completely superimposable, even though the birth weights differ by 15,000-fold, and the time scales by greater than 10-fold. However, the later linear growth is variable in relation to the underlying Gompertz curve, as indicated by the fanning out of this portion of the normalized curves of Figure 145. The data for the hamster show no linear component; the species for which an appreciable linear process has been measured include the cow, goat, mouse, rat, guinea pig, chicken, domesticated breeds of turkey, goose, and quail.

CONCLUSION

In this study we have interpreted the postnatal growth curves of various birds and mammals as potentially compound curves in which two components are distinguishable: an exponential growth process, which

FIG. 145.—Normalized plots of compound growth curves fitted to postnatal growth data of six mammalian species, selected to illustrate the variability in the linear component of the postnatal growth curve. The body weights were normalized by dividing by the computed asymptote of the Gompertz portion of the curve. The time scale was normalized by fixing the inflection point of each Gompertz curve at zero, and the unit for the time scale was set equal to the time in days required for the doubling of body weight immediately preceding the inflection point. The lines are the calculated compound curves giving the least squares fit to the data.

undergoes an exponential decay with increasing age (the Gompertz growth process), and a linear growth process, which yields a straight-line pattern in that part of the growth curve where little exponential growth is occurring. In biological terms, exponential growth, with or without a decay in time, is a process in which the products of growth reproduce themselves

and thereby contribute to the further growth of the organism. Linear growth in biological terms is accretionary growth, in which the products accumulate passively, without contributing further to the growth of the organism.

Evidence for the compound nature of postnatal growth rests on the observation that the rate of the

linear process can differ considerably in its relation to the underlying Gompertz process. It is possible that in domestication, animals are sometimes selected for the growth characteristics that result from exponential growth and sometimes for growth characteristics that are accretionary.

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NEUTRON THERAPY OF CANCER: BIOCHEMICAL AND BIOPHYSICAL ASPECTS

Norman A. Frigerio, Michael J. Shaw, and Hugh N. Nellans

PURPOSE AND METHODS

Three applications of neutrons to the problem of cancer therapy have been proposed:⁽¹⁾ fast neutron therapy, neutron capture therapy, and neutron conversion therapy. Of these the latter two involve biochemical, as well as biophysical problems. We have continued our efforts to provide effective chemical agents for these by localization and distribution studies, in normal and tumor-bearing animals, of compounds suggested by theory.⁽¹⁾ Where necessary, studies have been implemented by synthesis of unavailable compounds and by development of analytical techniques applicable to the determination of the nuclides of interest, ⁶Li, ¹⁰B, and ²³⁵U. At the same time we have attempted to ascertain the biological effectiveness of the intermediate energy neutrons proposed for therapeutic use—this by straight-forward studies of mouse lethality induced by monoenergetic neutrons in the 0.01- to 1.0-MeV range.

PROGRESS REPORT

Neutron Capture Therapy

Successful capture therapy places three requirements on the administered compound:⁽¹⁾ (1) The tumor concentration of the capturing nuclide must be $\geq 3.5 \times 10^{-3}$ M. (2) This concentration must be maintained for a period of several hours at least, preferably several days. (3) During this period the blood concentration should not exceed 10% of that in the tumor.

In addition it would be desirable for the tumor concentration to be at least tenfold that in any other part of the body. In this way whole-body irradiation could be given so as to "seek and destroy" primary tumors and metastases, regardless of their location.

Following our earlier experimental and theoretical

work,⁽¹⁾ we prepared and tested a number of boron-substituted macromolecules, using normal mice and mice bearing spontaneous tumors as test objects. Decaborane-14, B₁₀H₁₄, was found to react with bovine serum albumin (BSA), with gelatin, and with polyvinylpyrrolidone (PVP) to give products containing high concentrations of bound boron. A 5% solution of BSA in water was added, for example, to 4.5 g B₁₀H₁₄ and the mixture stirred at room temperature for 1 hr. During this period the solution became yellow, a white precipitate formed, and hydrogen was evolved. Reaction was continued for another 52 hr, i.e., until gas evolution ceased. After reaction the mixture was suspended in 100 ml of 0.15 M phosphate buffer at pH 7.2, extracted with diethyl ether, warmed to 40°C, centrifuged, filtered through a Millipore filter (0.8 μ), and dialyzed against distilled water at 0°C to constant conductivity. Part of the resultant solution was concentrated to 77 mg cm⁻³ with Sephadex gel and brought to 0.154 M in NaCl. The rest was lyophilized to constant weight and subjected to elemental analysis. Results for this (V—BSA), and other, similar, preparations, are shown in Table 56. Solutions of these compounds were injected intravenously into C3Hf/Anl (ANL 66) mice bearing spontaneous tumors of all sorts, e.g., tumor-bearing mice simply culled from our older stock animals. This procedure was used as being more representative of the clinical, human situation than implanted, or even induced, tumors would be. Results are shown in Table 57. Mice were sacrificed serially, organs and tumors dissected and analyzed. Concentrations given are averages over many animals. Standard deviations were never more than $\pm 10\%$, usually much less. Amounts injected were not limited by toxicity but, rather, by the maximum volume tolerable as an I.V. injection (0.20 ml). Indeed, no evi-

TABLE 56. REACTION OF DECABORANE-14 WITH BSA, PVP, AND GELATIN

Compound	B ₁₀ H ₁₄ , g	Mole ratio B ₁₀ H ₁₄	Reaction time, hr	Moles H ₂ /mole B ₁₀ H ₁₄	% B, w/w	Aqueous system solubility ^(a)
I—BSA	1.9	200:1	72	1.36	—	acet-water
II—BSA	6.1	500:1	360	12.52	1.53	insoluble
III—BSA	0.22	120:1	24	1.66	1.80	s
IV—BSA	3.05	500:1	51	2.64	—	b
V—BSA	4.5	500:1	53	6.35	3.10	b,s
VI—PVP	9.3	500:1	18	—	7.96	b,s, water
VII—Gelatin	3.0	ca. 500:1	168	>13.4	10.88	b,s, water

^(a) acet-water = water-acetone mixture; s = physiological saline; b = 0.15 M phosphate buffer at pH 7.2.

TABLE 57. LOCALIZATION OF BORON SUBSTITUTED MACROMOLECULES IN SPONTANEOUS MOUSE TUMORS

Product.....	V— BSA	V— BSA	V— BSA	V— PVP	VIII— Gelatin
Time after inj., hr.....	24	48	72	75	24
Amount inj., mg.....	15.4	15.4	15.4	38	29.5
Blood conc., ^(a) × 10 ⁻³	1.010	0.738	0.101	0.041	0.073
Skin ^(a)	0.30	0.138	0.168	0.247	0.142
Liver ^(a)	1.10	1.405	0.572	0.349	0.076
Kidney ^(a)	0.384	0.434	0.130	0.104	0
Brain ^(a)	0	0.120	0	0.110	0.260
Muscle ^(a)	0.126	0.070	0.30	0	0.082
Spleen ^(a)	0.433	0.528	0.215	0.130	0.039
Intestine ^(a)	0.122	0.070	0.090	0.139	0
Heart ^(a)	0	0	0.075	0.131	—
Whole tumor ^(a)	0.379	0.414	0.175	0.168	0.132
Tumor edge ^(a)	0.597	0.265	—	0.191	0.095
Tumor center ^(a)	0.441	0.513	—	0.116	0.068
Lungs ^(a)	—	—	—	0.308	0.348

^(a) Concentrations are given as moles B per kg fresh weight of tissue.

dence of toxicity was found at any achievable level, even on multiple injection.

With respect to requirement (1), the failure of the tumor concentrations (Table 57) to reach 3.5×10^{-3} M is not too serious. It is probable that these could be achieved by multiple injections spaced a few hours apart. As to (2), concentrations were well maintained for many hours. As to (3), tumor-to-blood ratios ranged from about 1.8 (BSA and gelatin) to 4.1 (PVP), so that further refinements of synthesis, purification, and injection kinetics should make it possible to achieve 10 or more. However, tumor-to-other-tissue ratios were disappointing, being less than one for such critical tissues as skin, liver, lung, kidney, etc. A few exploratory experiments suggested that this was due to formation of microaggregates of product via "denaturation" by the B₁₀H₁₄ during reaction. Since such denaturation appeared to be inevitable with

B₁₀H₁₄, we turned to the coupling of other boron moieties. B₉H₁₃NCS⁻ was synthesized by modifications of the method of Muettterties⁽²⁾ and coupled nicely to BSA. The product had a toxicity too low for detection by the methods available to us and contained 4.52% B. Preliminary tumor blood ratios were around 8.

Carboranyl aniline was prepared, B₁₀H₁₀C₂·HC₆H₄NH₂, and successfully diazotized to BSA. The product was so highly substituted (16% B), however, as to be water insoluble. Carboranyl aniline with an arsonic acid solubilizing group is being prepared and should give a more tractable product.

Phosphonic acids have a potential for tumor localization which has been neither tested nor exploited. They should bind well to proteins and, thus, localize by a mechanism similar to that of the sulfonic acids.⁽¹⁾ In addition phosphate anion is known to localize avidly in tumor cells.⁽¹⁾ If a single substitution of the anion is tolerated by the phosphate-uptake system of the tumor cell, then phosphonic acids might well prove the most potent localization agents of all. We have recently synthesized carboranyl phosphonic acid, B₁₀H₁₀C₂HPO(OH)₂, and hope to test it soon in tumor-bearing mice.

In pursuit of a useful ²³⁵U compound we have examined the toxicology of several U(IV) and U(VI) complexes. With the exception of uranyl phthalocyanine U compounds have, so far, proved too chemically toxic for therapeutic consideration.⁽¹⁾ Regardless of the complex or compound used, oxidation, hydrolysis, olation, and/or ionization have always resulted in the release of toxic amounts of uranyl ion. This year we examined three uranium complexes of great chemical stability, U(IV)-EDTA-TIRON,⁽³⁾ tetrakis tropolono U(IV), and lithium pentakis tropolono U(IV).⁽⁴⁾ Toxicity was about that of an equivalent quantity of uranyl ion and, on autopsy, the animals showed all of the characteristics of acute uranyl poisoning. As before chemical criteria of stability are simply not rigorous enough to serve as biological guides. The enzymes of liver and kidney

are better complexing agents than the best we are able to synthesize and easily win the uranium in a chemical competition, despite the Pyrrhic nature of the victory.

Neutron Conversion Therapy

^6Li seems the only useful nuclide for conversion therapy, by reason of its low toxicity.⁽¹⁾ In consequence we studied the toxicity, excretion, and distribution of Li^+ in normal and tumor-bearing animals. These were injected with LiCl , sacrificed serially, and their tissues analyzed for Li^+ by flame photometry. Some of the results obtained are shown in Figures 146 and 147.

Li concentrations of the order of $15 \times 10^{-3} M$ were easily obtained in all tissues immediately after injection, with the exception of the brain. Brain concentration was quite low, about $2 \times 10^{-3} M$. In consequence, tumor-to-brain ratios of about 7.5 were obtained. While this is not germane to conversion therapy *per se* it throws an interesting light on capture therapy using ^6Li and on early reports of the seeming localization of Li in brain tumors.⁽⁵⁾ Tumor-to-blood ratios of about 1.5 could also be obtained. While this is not important to conversion therapy it would add a certain degree of capture therapy "bonus" to conversion attempts using ^6Li .

In the absence of added NaCl , Li^+ cleared rapidly from the animal with an initial half-time of about an hour, followed by a slow phase with a half-time of >6 hr. If 1.0 to 2.0 moles of Na per mole of Li were included in the injection solution, a larger quantity of Li could be injected without toxic manifestations.

FIG. 147.—Li concentrations in other tissues as a function of time after injection. ○, Blood; ▲, brain; △, heart; □, kidney; ●, liver; ◐, muscle. Points represent the means of three values of Li concentration at 14.4 and 19.4 mM/kg.

However, competition of the two ions prevented increased Li concentration in the tumors, so that addition of Na provided little overall therapeutic advantage.

All in all, results were both encouraging and conclusive, so that future studies of this nature will utilize animals closer to man.

Neutron Biophysics

From a clinical standpoint it is necessary to convert the physical doses involved in the therapeutic use of neutrons into biological ones. We, therefore, initiated a study of mouse lethality as a function of neutron energy. This endpoint was chosen on the basis of its direct pertinence to the clinical problem. In addition, with the help of computer programs,⁽⁶⁾ extrapolation of lethality data to other problems becomes feasible.

Data can be accumulated only slowly because of the low dose rates obtainable from our present machines. This will be alleviated when the Physics Division high-current Dynamitron is in operation. Meanwhile, we have been able to increase present machine current by modifications to the source, slits, analyzer, flight tube, beam optics, etc. In addition, computations⁽⁶⁾ were made of the complex neutron space-energy distribution involved in the irradiation of several mice from a single, finite, angle-dependent source. These permitted intelligent design of a new 8-mouse "ferris wheel" of markedly increased irradiation efficiency. Preliminary results are given in Table 58. As with any neutrons below 0.7 MeV or so, problems of geometry, dosimetry, scatter, absorption, LET distribution, etc. are quite complex, even for so small an object as a mouse. These have been resolved by the combination

FIG. 146.—Li concentration in murine tumors as a function of time after injection. ▲, $14.4 \times 10^{-3} M$ Li injected per kg tissue; △, $14.4 \times 10^{-3} M$ Li plus $30.0 \times 10^{-3} M$ Na; □, $19.4 \times 10^{-3} M$ Li plus $30.0 \times 10^{-3} M$ Na; ○, $23.4 \times 10^{-3} M$ Li plus $30.0 \times 10^{-3} M$ Na.

TABLE 58. LETHALITY OF MONOENERGETIC NEUTRON EXPOSURE FOR MICE

Geometry	Neutron mean energy, keV	Dose mean LET, keV μ^{-1}	LD _{50/30} , rads	RBE ^(a) to $^{60}\text{Co } \gamma$
Therapy	50	70	250 $\pm$ 8	3.6
Single	390	90	185 $\pm$ 9	4.8
Single	555	40	305 $\pm$ 5	2.9
Conical	470	70	260 $\pm$ 8	3.5

^(a) LD_{50/30} for these mice was 900 rads for $^{60}\text{Co } \gamma$ and 680 rads for 250-KVP X rays. Thus RBE of X rays would be 0.76 the RBE given above.

of data from absolute flux-energy determinations as a function of laboratory and center-of-mass coordinates with absorbed dose data derived from tissue-equivalent and C-CO₂ or Al-Ar chambers used with and without tissue-equivalent phantoms. The needed data reduction was then done with the aid of a computer program described elsewhere.⁽⁶⁾

For Table 58 the $^7\text{Li}(p,n)$ reaction was used exclusively, as described elsewhere.⁽⁷⁾ Mouse irradiation geometry, however, was of several types. In "therapy" geometry, mice were exposed singly while imbedded in a human phantom at the maximum dose reference point.⁽¹⁾ In "single" geometry, mice were exposed singly, supported in free space along the 0° laboratory axis of the beam. In "conical" geometry, 8 mice were exposed at once along an isodose surface at a laboratory angle of 38.5°.

From the standpoint of therapy, the most important observation was that of an RBE not exceeding 3.6 for the 50-keV neutrons which we have proposed for therapy. Since the RBE's of the ^6Li , ^{10}B or ^{235}U capture reactions are certainly no less than 3.6 themselves, therapeutic computations, based on the physical rad dose⁽¹⁾ are, in fact, conservative.

From the radiobiological standpoint, it is of interest that the points obtained agree well, on an LET basis, with those obtained for mammalian cells *in vitro*. If this trend can be carefully confirmed in the 0.01- to 1.0-MeV neutron energy region, it will provide a powerful basis for the prediction of neutron radiobiological effects under any conditions.

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ULTRASTRUCTURE OF THE MURINE ASCITES TUMOR L4946

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PURPOSE AND METHODS

The ultrastructure of L4946 murine ascitic leukemia cells was studied; these tumor cells were predominantly lymphocytic. Samples of ascitic fluid were taken from CF- $\times$ 1/Anl (Anl 66) female mice at 3, 4, 7 and 10 days after they were inoculated with the tumor. The samples were centrifuged, and the cells were fixed in 2% OsO₄ with 2 mM CaCl₂, or in veronal acetate buffered 1% OsO₄ in 2 mM CaCl₂. Dehydration was in ethanol followed by propylene oxide. The cells were embedded in Epon 812, sectioned, and stained with uranyl acetate. An RCA-EMU-3F electron microscope was used to study their ultrastructure.

PROGRESS REPORT

Type A virus particles, similar in appearance to those described in Ehrlich ascites cells,⁽¹⁾ were typical of

L4946 leukemia cells (Figure 148, V). Intracisternal Type A virus particles (60-200 m μ in diameter) were associated primarily with the endoplasmic reticulum, and occasionally with the outer membrane of the nuclear envelope.

One sample contained intranuclear clusters of round, opaque, polyoma-like particles 35 m μ in diameter (Figure 149, V_n) in addition to the cytoplasmic virus particles.

No mitoses were observed electronmicroscopically, although several hundred leukemia cells were studied at magnifications up to 20,000 $\times$. However, an occasional mitotic cell was found by light microscopy in stained smears of 3- and 4-day-old samples. Mitotic cells were less frequently observed in samples taken later than 4 days. The nuclei of these ascitic leukemia cells were lobulated. Tufts of microfilaments were

FIG. 148.—Cytoplasm of L4946 ascitic leukemia cell. V, Type A virus particles in the endoplasmic reticulum (ER); Mf, microfilaments; M, mitochondria; R, ribosomes; $\times 37,000$.

frequently seen with the electron microscope at the constricted portions of the nuclear lobes. (Figure 150, T). These tufts extended into the cytoplasm from interruptions in the nuclear envelope. Electron-opaque particles, the size of ribosomes, were often observed at the bases of these tufts. Typical centrioles were seen (Figures 151 and 152, C). Other cytoplasmic components of the L4946 ascitic cells, typical of other mammalian cells, included Golgi bodies, mitochondria, microfilaments, ribosomes, annulate lamellae, and clusters or aggregates of vesicles.

CONCLUSIONS

L4946 ascites leukemia cells contain virus particles typical of other rodent tumors.⁽²⁻⁴⁾ These particles appear to begin their development on the cisternae of the endoplasmic reticulum as electron-opaque thickenings.

These opaque areas presumably invaginate and grow into spherical forms. They appear to mature into the well-developed virus particles observed in this tumor cell.

Mitoses occur with high frequency (mitotic index of 3 to 5%) about 1 day after inoculation of tumor cells

FIG. 149.—Intranuclear virus-like particles (V_n) in an ascitic leukemia cell. N, Nucleus; V, Type A virus particles in the endoplasmic reticulum (ER); Mf, microfilaments. $\times 29,000$.

FIG. 150.—T, Tuft of microfilaments interrupting the nuclear envelope at the constricted neck of nuclear lobe; N, nucleus; C, cytoplasm. $\times 68,000$.

FIG. 151.—Cross section of a centriole (C) surrounded by Golgi bodies (G) and nucleus (N); Ve, vesicle aggregate. $\times 46,000$.

into the mouse peritoneal cavity. This index is approximately 1 to 2% at day 3, and becomes less at day 4.

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FIG. 152.—Longitudinal section of two adjacent centrioles (C) and their satellites (S); N, nucleus; Ve, vesicle aggregate. $\times 53,000$.

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METABOLIC AND THERAPEUTIC STUDIES OF PLUTONIUM. III.

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Martin H. Smoler, and Elizabeth S. Moretti

PURPOSE AND METHODS

This continuing program on the therapy of poisoning by radioactive and nonradioactive metals⁽¹⁾ has placed increasing emphasis upon plutonium.^(2, 3) The biological aspects of plutonium are of both practical and theoretical interest. First, from the radiological health aspect, plutonium-239 can be considered the number one potential hazard because of the magnitude of its projected use in public power reactors. Second, it is a bone-seeker, highly effective in the induction of malignant bone tumors. Third, plutonium is useful for basic studies of the physiological behavior of colloids and macromolecules in animal tissue; its compounds readily hydrolyze and polymerize, both in solution and in animal tissues, to form labeled colloids and particulates whose translocation in animal tissues can be followed.

Better understanding of the biological behavior of this element is expected to lead to the development of improved therapeutic procedures, both for plutonium and for other polyvalent, easily hydrolyzed heavy metals, including thorium, uranium, and americium. From a therapeutic point of view, the plutonium in two biological compartments is of particular concern: (1) the fraction of plutonium in the skeleton that is not readily removed by chelation therapy with diethylenetriaminepentaacetic acid (DTPA), and (2) polymerized plutonium in the liver, which is also inaccessible to DTPA.

Related to the plutonium program is a concomitant program⁽⁴⁾ concerned with the normal processes of calcium uptake and depletion and the biochemical transformations in the calcifying matrix of preosseous cartilage. This study is designed (1) to provide a better understanding of the uptake of metal cations, including such diverse radionuclides as plutonium and strontium, in cartilage and bone and (2) to suggest new approaches to the therapeutic removal of toxic metals from these tissues.

PROGRESS REPORT

Therapy of Plutonium Poisoning

Long-Term Effects.—Removal of either polymeric or monomeric plutonium from the bone of mice by

DTPA therapy significantly increases the survival time and the latent period before bone tumor development; it also decreases the rate of death with tumors, as well as the lifetime incidence of bone tumors.^(5, 6) Further, the lifetime bone tumor incidence (percent of mice with tumors) is higher after injection of monomeric than after polymeric plutonium. The long-term effects of the two forms of plutonium have now been compared more exactly, using as the basis of comparison both the bone burden of plutonium 15 days after injection and the distribution of plutonium on bone surfaces and in marrow. The results of this comparison⁽⁷⁾ emphasize that the physical form (monomeric or polymeric) in which the plutonium is transported in the blood and in which it reaches the skeleton has a significant effect on the microdistribution of the plutonium in bone and marrow, and, eventually, on its carcinogenic effect. With equal amounts in the bone, monomeric plutonium is at least twice as carcinogenic as polymeric plutonium, presumably because of more radiation delivered to the cells at risk near bone surfaces.⁽⁸⁾

The preceding long-term studies with two forms of plutonium have now been extended, in conjunction with Dr. Hermann Lisco of New England Deaconess Hospital, to include determination of the numbers of tumors per mouse and a detailed histopathological examination of the bones. The following tentative conclusions can be made: After injection of monomeric, compared to polymeric plutonium, there were (a) the same proportion of mice with only one tumor, (b) more mice with multiple tumors, and (c) only 6% vs. 24% of the mice with a peculiar generalized bone pathology. This condition was more common in tumor-bearing mice than in mice without tumors; the probability of its occurrence increased with the number of tumors per mouse.

In conjunction with these studies of plutonium-induced bone tumors in mice, a detailed analysis has been made of the plutonium content throughout the skeleton, with and without DTPA therapy, in order to relate location of bone tumors to that of plutonium. After injection of polymeric plutonium, the amount deposited in each bone, with a few exceptions,

was approximately two-thirds that found after injection of monomeric plutonium. DTPA removed either form of plutonium from each bone to about the same extent.

Plutonium and the Reticuloendothelial System (RES).—The RES stimulant, glucan, when administered after injection of a polymeric form of plutonium, leads, within one month, to an increased loss of plutonium from the liver.⁽³⁾ The removal of plutonium from the liver by glucan, combined with continuing chelation therapy with DTPA, is nearly additive. Quantitative autoradiographic studies of the livers of these mice have shown that the removal of hepatic plutonium by either agent was accompanied by a reduction in the concentration of single alpha tracks and of stars, the latter representing larger aggregations of plutonium. Both treatments, either alone or combined, caused a greater reduction in the plutonium of the parenchymal than of the littoral areas. Under the influence of glucan, the aggregates of plutonium in the liver appear diffuse, as if the plutonium particles have either been separated or kept from aggregating closely by intervening, nonradioactive substances. These substances have been identified by PAS staining as probably glucan or polysaccharide derivatives of glucan. Evidence is presented⁽⁹⁾ that these more diffuse aggregations of plutonium are easily dissociated by DTPA or through natural metabolic processes and removed via the circulation.

Another new approach to the removal of polymeric plutonium from liver has involved the use of specific tissue antigens, obtained from rabbits injected with mouse RES tissues, to liberate intracellular plutonium in the mouse RES tissues, particularly those of the liver. Rabbit antisera to either mouse spleen protein or to mouse peritoneal cell protein have been obtained. Preliminary experiments in mice previously given polymeric plutonium suggest that these antisera may enhance the DTPA removal of plutonium from the liver and that, without DTPA, they may induce translocation of plutonium to the spleen and possibly the bone. The optimum conditions for this therapeutic approach are being determined.

Experimental Modification of Bone Metabolism.—As an adjunct to chelation therapy, the increase in osteoclast numbers induced by parathyroid hormone (PTH)⁽¹⁰⁾ is being utilized in an attempt to liberate plutonium sequestered on bone surfaces. Repeated injections of a low level of PTH (50 units per day of Lilly parathyroid extract for 11 days) were given to weanling male rats injected with monomeric plutonium (94% ultrafilterable) 6 days previously. Seventeen days after plutonium administration, the PTH had no effect on the plutonium content of the femurs,

liver, or spleen. Neither did it alter the removal of plutonium from these tissues by concomitant therapy with DTPA (200 mg/kg for 11 days). The control rats lost a significant fraction of the bone burden of plutonium between 6 and 17 days. Although results with this therapeutic regimen are not promising, other variations of hormonal manipulation are being considered.

Studies of Particulate Plutonium in Vivo

The need for carefully defined radiocolloids in basic and therapeutic studies has been emphasized by earlier work.^(2, 3) Plutonium solutions of narrow particle size range have been prepared by passage through Sephadex.⁽¹¹⁾ In the preparation and characterization of plutonium particulates, the following arbitrary designations are now given to solutions prepared for biologic work: Solutions which contain a large variety of particle sizes are referred to as *ungraded* solutions. Fractionated solutions, which contain a narrower range of particle sizes, are termed *graded* solutions. Solutions of either type, in which the fraction of ultrafilterable plutonium is 25–70%, are designated as *mid-range* solutions.

The gross tissue distribution in mice following a variety of such carefully characterized plutonium solutions has been determined; in addition, studies have been made at the cellular level, using a combination of quantitative autoradiography and light microscopy.⁽¹¹⁾ It was found that an unexpectedly large fraction (over one-half) of the liver plutonium was associated with parenchymal cells following injection of any form of plutonium. This work is being extended to the subcellular level by a combination of autoradiography and electron microscopy.

Techniques for Radioactivity Measurement

The use of solid state detectors for measuring alpha emitters, such as plutonium and americium, is being explored in collaboration with Mr. William Eisler of the Instrument Group in the Division of Biological and Medical Research. These detectors offer such advantages as low backgrounds and the possibility of discriminating among mixtures of radionuclides emitting particles of different energies.

A liquid scintillation procedure for measuring plutonium alpha radiation⁽¹²⁾ is now being used routinely in the assay of plutonium in mouse tissues. A modification of the technique used for mouse tissues now permits the measurement of plutonium in the presence of greater amounts of interfering substances, as in the larger organs of the rat. In connection with this work, it has been found that the distribution of polymeric plutonium in the rat liver, at 5 days after injection,

is sufficiently uniform to allow the use of a weighed portion of liver tissue for plutonium assay.

CONCLUSIONS

The results of long-term studies in mice relating bone tumor incidence to gross and microscopic deposition of plutonium in bone indicate that the maximum permissible bone burden for plutonium, and presumably also for any other hydrolyzable alpha-emitting radionuclide, should be based on its microscopic location in bone, as well as on the total bone burden. The microdistribution pattern of plutonium in bone and marrow, and subsequently, the lifetime incidence of bone tumors, have been shown to be dependent upon the particulate characteristics of the administered plutonium.

The mode of entry of plutonium into the body after an accidental exposure may also affect its tissue distribution and carcinogenic properties by influencing the form of the plutonium translocated from the original site of deposition. For example, plutonium accidentally introduced into skin or muscle, but probably not into lung, is translocated to the skeleton as monomeric plutonium,⁽⁶⁾ and would be expected, therefore, to be more carcinogenic than if translocated to bone as a polymer.

The results with quantitative autoradiography support previous evidence^(3, 13) suggesting the uptake of plutonium by hepatic parenchymal tissue; more than one-half of the hepatic plutonium, after injection of any form, was found to be associated with parenchymal cells rather than littoral (RES) cells. The retention of polymeric plutonium in the liver, up to at least 3 months, is accompanied by a progressive aggregation of plutonium-containing material. Quantitative localization of plutonium in animal tissues at the cellular and subcellular level may be useful for microdosimetric calculations.

The constancy of the fraction of the total skeletal burden of plutonium in each individual bone has practical value in the estimation of total skeletal burden from single bone samples, as obtained, for example, from human autopsies.

A new therapeutic approach utilizes glucan, a relatively nontoxic material, to remove a significant fraction of polymeric plutonium from the liver that is not available to the chelating action of DTPA. The posi-

tive results obtained suggest exploration of the therapeutic effects of other polysaccharides.

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BIOCHEMISTRY AND ION BINDING OF CONNECTIVE TISSUE. II.

Arthur Lindenbaum, Carol J. Lund, and Martin H. Smoler

PURPOSE AND METHODS

In the removal from the body of many toxic radioelements, including the polyvalent, heavy metals, such as plutonium, bone is the critical tissue. A better understanding of normal and abnormal physiological processes in bone, therefore, is of practical as well as fundamental importance. The objective of this project is to investigate the biochemical changes taking place in cartilage during calcification and ossification, particularly with regard to changes in the mucopolysaccharide and mucoprotein components, and with involvement of such substances in the accumulation of metal cations. Previously developed techniques are being utilized and amplified, using the dog and calf scapula as the experimental tissue.

PROGRESS REPORT

Earlier work has utilized the sharply defined developmental regions of the ossifying calf scapula to isolate and characterize the organic components, particularly the mucopolysaccharides and mucoproteins, of the cartilage zones.⁽¹⁻⁹⁾ At present, the differences previously noted between the components of resting and ossifying cartilage are being investigated from two different points of view. In the first, a histological study, using both light and electron microscopy, is under way. The objective is to correlate structural changes at the cellular and subcellular levels in cartilage and bone with previously noted changes in chemical constitution and enzymatic activity. The second approach involves preliminary work aimed at measuring the cation binding properties of mucoproteins isolated from cartilage areas representing different stages in the transformation of ossifiable cartilage.

CONCLUSIONS

Although the above portions of this work are in a very early stage, there are already indications that further efforts are worth while. For example, electron microscopy has already revealed unusual structures

in the columnar cells of the transition region between resting and ossifying cartilage; these have not been seen under the light microscope. Also, the improved resolution, provided by electron microscopy, of subcellular details in hypertrophying columnar chondrocytes near the cartilage-bone junction should permit a more precise determination of the drastic changes undergone by these cells and, when combined with quantitative autoradiography, of the localization of deposited cations (including fission radionuclides) at the cellular and subcellular levels.

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AN AUTORADIOGRAPHIC STUDY OF THE CHANGES IN DISTRIBUTION OF POLYMERIC PLUTONIUM IN MOUSE TISSUES*

Arthur Lindenbaum, Marcia W. Rosenthal and Martin H. Smoler

Mice injected intravenously with 0.2 μ Ci of polymeric plutonium were sacrificed at intervals between $\frac{1}{2}$ hr and 89 days. The distribution and localization of plutonium alpha activity in kidney, spleen, liver, and bone marrow were determined by quantitative autoradiography. In the kidney, up to 6 hr after injection of plutonium, there was four times as much plutonium in the glomeruli as in the tubules. As ob-

served up to 5 days in the spleen, the plutonium was concentrated in increasing amounts in the perfollicular zones of the red pulp. In the liver, about 60% of the tracks were associated with parenchymal cells and 40% with littoral cells at all intervals from $\frac{1}{2}$ hr to 89 days. Progressive aggregation of plutonium in liver was indicated by the appearance of stars between 1 and 5 days, and by an increase in the size of stars thereafter. The plutonium concentrations in the liver, based on track counts, were in agreement with those obtained by gross radiochemical assay.

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PREPARATION, CHARACTERIZATION, AND DISTRIBUTION IN MOUSE TISSUES OF GRADED POLYMERIC AND MONOMERIC PLUTONIUM. RADIOCHEMICAL AND AUTORADIOGRAPHIC STUDIES*

Arthur Lindenbaum, Carol J. Lund, Martin H. Smoler, and Marcia W. Rosenthal

Solutions of plutonium(IV) nitrate, adjusted to pH 5.5-6.0 and stabilized with citrate, were fractionated with respect to particle size by passage through Sephadex columns. Three graded solutions of narrow particle size range thus obtained (in which the fraction of plutonium ultrafilterable through cellophane membranes was <1%, 30%, and 100%, respectively) were injected intravenously into mice. At sacrifice, 5 days later, livers, spleens, femurs, and tibial shafts (the latter separated into hard bone and marrow fractions) were removed for plutonium analysis and autoradiography. With increasing ultrafilterability, less plutonium was deposited in liver, spleen, and marrow, while more was deposited in bone. Injection of the graded solution containing 30% ultrafilterable plutonium, from which both larger and smaller par-

ticles had been removed, resulted in a significantly different organ distribution of plutonium as compared with the ungraded solutions of the same ultrafilterability previously used. With the graded solutions, the greatest differences in tissue deposition were found between the most polymerized plutonium and the two less polymerized forms. The pattern of deposition in mouse tissues thus appears to depend on aggregation of plutonium-containing particulates to some critical particle size. Filtration of plutonium solutions through calibrated Millipore filters indicates that this critical size is below 0.5 μ and well above 0.01 μ in diameter. Quantitative autoradiography of livers from mice given graded polymeric plutonium showed differences in deposition between parenchymal and littoral cells (60% vs. 40%, respectively); the ratio was larger (70% vs. 30%) after the graded monomeric solution. Stars in both liver and spleen were seen only after the most polymerized solution.

* Abstract of paper to be published in the Proceedings of the Symposium on Diagnosis and Treatment of Deposited Radionuclides, May 15-17, 1967 (Excerpta Medica).

COMBINED RETICULOENDOTHELIAL STIMULATION AND LONG-TERM, INTERMITTENT DTPA THERAPY IN POISONING BY POLYMERIC PLUTONIUM*

Marcia W. Rosenthal, Martin H. Smoler, and Arthur Lindenbaum

Mice were given a single intravenous injection of 0.1 or 0.2 μCi of polymeric plutonium, about 30% ultrafilterable, and ungraded as to particle size. Beginning 5 days later, they were given 40 mg/kg of the reticuloendothelial stimulant, glucan, once daily for 3 days, or 500 mg/kg of DTPA, once every 3 days for 28 injections, or they were given both treatments. In mice given glucan alone, there was no effect on the plutonium content of any tissue at the height of the reticuloendothelial stimulation. Near the end of the period of stimulation (41–49 days after plutonium), the liver content of plutonium was lower (by about 10% of the injected dose) than the level in the controls, and the bone content had increased by about the same amount. In mice given DTPA alone, the

liver content of plutonium was reduced by about the same amount, and the bone burden was reduced to 30% of the control level by 89 days. Combined, the two treatments had a nearly additive effect in reducing the liver burden and had the same effect in bone as DTPA alone. Autoradiograms of livers showed that both glucan and DTPA reduced the concentration of plutonium in the hepatic parenchyma to a greater extent than they did in the sinusoidal areas. Concentrations of single alpha tracks and of stars, representing larger plutonium deposits, were reduced. In addition, in glucan-injected mice, either with or without DTPA, the plutonium aggregates appeared more diffuse and disorganized than normal. These results suggest that these glucan-induced aggregates break up easily and permit more plutonium to be removed either by natural mechanisms or by therapeutic agents such as DTPA.

* Abstract of paper to be published in the Proceedings of the Symposium on Diagnosis and Treatment of Deposited Radionuclides, May 15–17, 1967 (Excerpta Medica).

OSTEOSARCOMAS AS RELATED TO TISSUE DISTRIBUTION OF MONOMERIC AND POLYMERIC PLUTONIUM IN MICE*

Marcia W. Rosenthal and Arthur Lindenbaum

The long-term effects of ungraded, midrange polymeric plutonium (about 66% ultrafilterable) and of monomeric plutonium (93% ultrafilterable) intravenously injected into mice are compared, using the bone burden of plutonium 15 days after injection as the basis of reference. Two levels of polymeric plutonium in the total skeleton (0.015 and 0.0087 μCi) and three of monomeric plutonium (0.025, 0.012, and 0.0058 μCi) were compared, the lower levels in each

case resulting from DTPA therapy. Extrapolation of the results, to enable comparison of the effects of equal amounts of plutonium in the bone, showed that mice injected with monomeric plutonium, compared to those injected with polymeric plutonium (a) had at least twice as high an incidence of osteosarcomas (twice as many mice with tumors and more tumors per mouse); (b) died with osteosarcomas at approximately the same rate after the latent period; and (c) survived approximately as long. Differences in the microdistribution of the plutonium on bone surfaces and in marrow are considered primarily responsible for these differences; other possible factors are also discussed.

* Abstract of paper to be published in the Proceedings of the Symposium on Delayed Effects of Bone-Seeking Radionuclides, Sun Valley, Idaho, September 11–14, 1967, Ed. Charles W. Mays (The University of Utah Press, Salt Lake City).

AUTORADIOGRAPHIC AND RADIOCHEMICAL STUDIES OF THE EFFECT OF COLLOIDAL STATE OF INTRAVENOUSLY INJECTED PLUTONIUM ON ITS DISTRIBUTION IN BONE AND MARROW*

Marcia W. Rosenthal, John H. Marshall,[†] and Arthur Lindenbaum

Radiochemical and autoradiographic evidence is presented showing that after intravenous injection of Pu into mice there is an appreciable deposition of

plutonium in bone marrow at 6 days and for at least 3 months. The amount of plutonium in the marrow, and its degree of aggregation, is greater, and the amount of plutonium on bone surfaces is smaller, after injection of polymeric than after monomeric plutonium. Although the amount of plutonium in the marrow is appreciable, there is always less in the marrow than on the bone surfaces.

* Abstract of paper to be published in the Proceedings of the Symposium on Diagnosis and Treatment of Deposited Radionuclides, May 15-17, 1967 (Excerpta Medica).

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MUCOPOLYSACCHARIDES AND MUCOPROTEINS OF CALF SCAPULA*

Arthur Lindenbaum and Klaus E. Kuettner[†]

The sharp division between cartilage and bone in the ossifying calf scapula was found to allow fractionation, in nearly unlimited quantities, of both bone and cartilage in progressive zones of transformation. The mucopolysaccharide (MPS) components of each zone were isolated by enzymatic degradation of tissue, followed by ethanol precipitation. Mucoprotein (MP) components were obtained by water extraction of cartilage homogenates below 10°. MPS isolated from three different cartilage zones, varying in distance from the cartilage-bone junction, consisted mainly of galactosaminoglycans of the chondroitin sulfate A and

C type. The highest concentrations of MPS and MP were found in the 2-mm-wide ossifying cartilage zone adjacent to the bone. There appears to be a shift toward MPS of higher molecular weight in this region. Titration of MPS isolated from each zone yielded a constant equivalent weight value of about 273. In contrast, titration of MP extracted from each cartilage region indicated a rise in equivalent weight, and a reduction in exchangeable sulfate, toward the cartilage-bone junction. Removal of coextracted serum proteins altered the MP values, presumably by making available functional groups which are covered up or occupied in the natural state. Microradiograms indicate that the radiopaque mineral in cartilage is localized mainly in the narrow zone, no more than 0.1 mm wide, immediately adjacent to the newly formed bone.

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COMPARISON OF GALACTOSAMINOGLYCANS ISOLATED FROM DIFFERENT REGIONS OF CALF SCAPULA CARTILAGE*

Klaus E. Kuettner[†] and Arthur Lindenbaum

The process by which cartilage in some areas of the body is transformed and eventually replaced by bone,

whereas seemingly similar cartilage in other areas remains unchanged, is of fundamental and practical interest. In preparation for investigations of the cation binding properties of ossifying cartilage, our attention has been directed to the mucopolysaccharide (MPS) and mucoprotein components. The MPS isolated from

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different developmental regions (zones) of calf scapula cartilage consists mainly of the galactosaminoglycans of the chondroitin sulfate A and C type. The present report describes further attempts to characterize and compare acid mucopolysaccharides, isolated by different techniques, from cartilage zones designated, in accordance with previously adopted terminology as "resting," "transforming," and "ossifying." In one experiment, mucopolysaccharides were isolated by fractional precipitation in increasing concentrations of ethanol, according to the technique of Meyer et al.⁽¹⁾ In a second experiment, the cartilage MPS was isolated as a single precipitate by the method of Greiling et al.⁽²⁾ After removal of interfering substances by dialysis, the equivalent weights of MPS isolated from each zone by either method were reasonably close to the theoretical value of 243.7. Although the isolated mucopolysaccharides of the three different cartilage zones do not differ significantly in their chemical

composition, they differ in their precipitability by ethanol. The ratios of the amounts of MPS in each zone precipitated in 28–33% ethanol and 33–40% ethanol (the two concentration ranges in which most of the MPS is isolated) were found to be 0.5, 0.4, and 1.8, respectively, for resting, transforming, and ossifying cartilage. These results suggest that in the cartilage area closest to the bone, there is a shift toward MPS of higher molecular weight. Shifts in molecular size without corresponding changes in equivalent weight of these macromolecular polyelectrolytes might significantly affect their total exchange capacity, and consequently alter their ability to bind cations.

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EFFECTS OF STARVATION ON THE ULTRASTRUCTURE OF AMOEBA MITOCHONDRIA

Edward W. Daniels and Evelyn P. Breyer*

PURPOSE AND METHODS

Pelomyxa carolinensis, a radioresistant species of amoeba, was originally obtained from North Carolina. Typically, mitochondria from this amoeba have randomly oriented tubules within the matrix (Figure 153). Zig-zag tubules in the mitochondria of *P. carolinensis* were first demonstrated by Pappas,⁽¹⁾ Pappas and Brandt,⁽²⁾ and Borysko and Roslansky.⁽³⁾

We sought the cause for this striking ultrastructural difference in mitochondria of *P. carolinensis* grown in our laboratory. We had found zig-zag tubules, like those described by Pappas, in the mitochondria of *P. illinoisensis*, a radiosensitive amoeba. In *P. illinoisensis*, there is a relationship between the induction of zig-zag mitochondrial tubules and the beginning of mitosis.⁽⁴⁾

Our present study has demonstrated that starvation induces zig-zag tubules in the mitochondria of *P. carolinensis*. We have extended our early exploratory work on starving amoebae⁽⁵⁾ and have discovered another ultrastructural change which is presented here.

Before initiating starvation, amoebae were selected from stock cultures and fed an excess of ciliates and flagellates. After one hour, all food organisms and most

of the bacteria were removed, and the amoebae were placed in clean glass containers of glass distilled water at pH 7.0, or in phosphate buffer at pH 6.8.⁽⁶⁾

Except for a few initial experiments, each amoeba, rather than groups of amoebae, was examined with the light microscope at the beginning of the starvation period to determine whether or not it was well fed and healthy. During the starvation period, all amoebae were observed daily, and their foodless environment was kept fresh. The glassware was routinely changed, and the aqueous medium was replaced. Most of the amoebae were starved at room temperature ($25 \pm 0.5^\circ\text{C}$), but a relatively small number were starved at 23°C in a constant temperature room.

Fixation was in 2% unbuffered osmium tetroxide with 2 mM CaCl_2 for one hour or overnight. Dehydration was in an ethanol series followed by propylene oxide. The amoebae were embedded in Epon, sectioned with a Du Pont diamond knife in either a Porter Blum or a Huxley microtome, and studied with an RCA EMU-3F electron microscope.

PROGRESS REPORT

In some of the mitochondria of starved amoebae, the tubules are aligned in a zig-zag fashion (Figure 154) instead of being randomly oriented in the matrix (Figure 153). Usually the zig-zag tubular patterns do not occupy more than half of the mitochondrion;

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the remainder is taken up largely by the matrix. The zig-zag tubules are larger in diameter than those of the mitochondria in well-fed amoebae, and there is relatively little matrix material between them (Figure 154, Mx). Thus, the matrix volume/intratubular volume ratio in the zig-zag areas of mitochondria is

FIG. 153.—Mitochondria from well-fed amoeba, *Pelomyxa carolinensis*. T, Tubules; Mx, matrix; O, outer mitochondrial membrane; I, inner mitochondrial membrane; C, cytoplasm. $\times 37,000$.

FIG. 154.—Mitochondrion showing zig-zag tubules from an amoeba starved 16 days. T, Tubules; Mx, matrix; B, blind end of tubule in matrix space; J, junction of tubular lumen with space between the two mitochondrial membranes. $\times 56,000$.

FIG. 155.—Mitochondrion showing microfilaments. From an amoeba starved 16 days at 23°C in buffer. Mf, microfilament bundles. $\times 33,000$.

FIG. 156.—Mitochondrion from an amoeba starved 16 days. Both zig-zag tubules and a microfilament bundle are shown. The gradual enlargement of two of the tubules as they enter the zig-zag area can be seen at arrows. $\times 46,000$.

markedly reduced. Zig-zag tubular patterns in the mitochondria of well-fed amoebae were absent.

In the starvation experiments, 81% of the amoebae that were starved from 1 to 21 days contained mitochondria with zig-zag tubules. The mean number of these mitochondria per amoeba increased as starvation was lengthened from 1 to 17 days. Peaks of increase occurred on the 3rd, 10th, and 16th days of starvation when percentages of mitochondria with zig-zag tubules, per amoeba, were 8%, 34%, and 79%, respectively. After the 16th day of starvation, the mean number of these mitochondria per 100 mitochondria in each amoeba studied was gradually reduced.

If starvation is extended to more than a few days, microfilaments are found in the mitochondrial matrix.

FIG. 157.—Zig-zag tubules and a cross section of two microfilament bundles are demonstrated in this mitochondrion from an amoeba starved 21 days, 16 hr. Mf, Microfilament bundles in cross section. $\times 48,000$.

FIG. 158.—Cross-section of a filament bundle in a mitochondrion from an amoeba starved 17 days. Mf, Microfilament bundle; Mx, matrix; T, cross section of tubule. $\times 73,000$.

These increase in number as starvation is continued, and occur in bundles (Figures 155 and 156, Mf). In addition, the percentage of mitochondria containing bundles of microfilaments increases as the starvation of an amoeba is lengthened.

These microfilaments appear solid in cross section (Figures 157 and 158, Mf) and might be crystalline.

All of the unfixed amoebae died after approximately three weeks of starvation.

CONCLUSIONS

The mitochondria of well-fed amoebae are characterized by randomly oriented tubules, instead of cristae, extending from the inner membrane of the mitochondrion into the matrix. These tubules are often branched, and their distal ends that lie in the matrix are closed. The region within the tubules is continuous with the area between the double membranes. Microfilaments and enlarged, zig-zag tubules are absent from the mitochondria of well-fed amoebae.

In starving *Pelomyxa carolinensis* amoebae, many mitochondria contain enlarged, zig-zag, parallel tubules, and microfilament bundles that lie within the matrix. Occasionally, both zig-zag tubules and microfilament bundles occur in the same mitochondrion. The zig-zag tubular design appears to be the result of tubular swelling and realignment. The microfilaments appear to condense out of the bulk of the matrix substance in the portion of the mitochondrion where zig-zag tubules are absent. The mechanisms that directly cause these ultrastructural differences in the mitochondria of starving amoebae are unknown. During starvation, the amoebae are forced to live initially on stored food supplies. Later, they probably metabolize protein and lipid from their own protoplasm. Presumably, mitochondrial enzymes are then required to make ATP from predominantly different precursors. It is our supposition that these biochemical changes, in some manner, induce the ultrastructural changes described in this report.

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EXTENSION OF THE DISTRIBUTION OF THE GIANT AMOEBA, *PELOMYXA CAROLINENSIS*

Edward W. Daniels and Patricia A. Sustarsic

PURPOSE AND METHODS

The giant amoeba, *Pelomyxa carolinensis*, was first found in North Carolina by Wilson in 1900.⁽¹⁾ It has also been found in Virginia, Tennessee, New Jersey, New York, and Illinois.⁽²⁾

In 1964, Dr. J. F. McClellan, Department of Zoology, Colorado State University at Fort Collins, discovered some giant, multinucleated amoebae resembling *P. carolinensis*. They were found near that university. From preliminary light microscopic and cultural study, he tentatively concluded that the amoebae were *P. carolinensis*.⁽³⁾ However, there were no publications on the classification of the new amoeba. Dr. McClellan kindly gave one of us (E.W.D.) a culture of these organisms in 1965.

Because of the broad interest in giant amoebae, and because of their usefulness in research and teaching, we decided to determine the proper classification of the Colorado amoebae. We compared their morphology and cultural characteristics with those of the three species of *Pelomyxa*, *P. carolinensis* from North Carolina, *P. illinoisensis*,⁽⁴⁾ and *P. palustris*.⁽⁵⁻⁷⁾ We used light and electron microscopes in our study, and also transplanted grafts of both *P. carolinensis* and *P. illinoisensis* protoplasm into the new amoebae from Colorado. Our microsurgical techniques were similar to those used previously.⁽⁴⁾ The microsurgery was done at a temperature of $25 \pm 0.5^\circ\text{C}$, but the amoebae were cultured thereafter at 23°C . The reason for this is that *P. illinoisensis* is sensitive to temperatures above 26°C .

The present work is being organized for publication, although a few experiments, not included in this report, are still in progress.

PROGRESS REPORT

The ultrastructure of *P. carolinensis* is similar to that of the Colorado amoeba.

The microsurgical transfer of protoplasm from each of 24 *P. carolinensis* amoebae was made into 24 different recipient amoebae from Colorado. As soon as a microsurgical transfer was completed, the resultant amoeba was isolated and cultured. These isolates were observed and cared for each day, and kept at 23°C . After 6 days from the time of grafting, each recipient amoeba had given rise to an average of ten offspring. Some of the cultures were kept for a month, and these amoebae continued to multiply during this time. This

transplantation tolerance is complete, and it indicates that the Colorado amoeba is identical to *P. carolinensis*. *P. carolinensis* is also identical to *Chaos chaos*, as shown in previous work.⁽⁴⁾

Heterologous transplantation⁽⁴⁾ of *Pelomyxa illinoisensis* amoeba protoplasm into the Colorado recipients was done on 13 different pairs of organisms. There were no subsequent cell divisions, and all the recipients died several days following the transplants. Controls from both donor and recipient species which were not used in microsurgery continued to grow and multiply. Thus, according to these data, the Colorado amoebae are not *P. illinoisensis*. The Colorado amoebae are clearly different from *Pelomyxa palustris* in cultural characteristics, body form, and ultrastructure.⁽⁵⁻⁷⁾

CONCLUSION

Collections of a giant amoeba resembling *P. carolinensis* were made in Colorado in 1964 by Dr. McClellan, Colorado State University at Ft. Collins. These amoebae have not been definitely classified, according to the absence of published literature and Dr. McClellan.⁽³⁾ The work presented here demonstrates that the Colorado amoebae completely tolerate *P. carolinensis* protoplasm that is injected into them by microfusion. On the other hand, they do not tolerate the protoplasm from *P. illinoisensis* amoebae injected in a similar manner. The data obtained indicated that the Colorado amoebae are *P. carolinensis* amoebae.

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EFFECT OF WEIGHTLESSNESS ON AMOEBAE

Edward W. Daniels and Donald R. Ekberg*

PURPOSE AND METHODS

The giant, multinucleated amoeba, *Pelomyxa carolinensis*, is being used to study possible effects of weightlessness on the ultrastructure of cells in a NASA supported space-effect investigation. One of our objectives is to determine whether or not the many nuclei in this amoeba, normally synchronized, become asynchronous in a weightless environment.

The method involves the use of the electron microscope to study amoebae that were fixed during and

acteristic of the Bios space vehicles during lift-off and impact, and they range from about 30 to 2,000 cps. Both random and sinusoidal vibrations were studied. The resonance frequencies of various sub-cellular structures were also investigated, and a part of this investigation involved the electron microscopy of *P. carolinensis* used in these test experiments. At the higher levels of *g* (8–12 *g*), fragmentation of the amoebae increased and some of the cells were ruptured. However, no ultrastructural differences in the survi-

TABLE 59. GLUTARALDEHYDE FIXATIVE PREPARATIONS FOR AMOEBAE

Concentration, %	Buffer	Time of fixation, days	Rinse	Post-fixation	Results
0.5	0.1 M Phosphate	1–2	None	Roth's fixative ^(a) 16 hr	Fair
2	0.05% Sodium cacodylate	4	0.05% Sodium cacodylate	"	Poor
3	0.1 M Phosphate + 1 mM CaCl ₂	2	0.1% Phosphate buffer + 10% sucrose	1% OsO ₄ 16 hr	Poor
3	"	"	0.1% Phosphate buffer	"	Nuclei good; nuclear envelope segmented; mitochondria aggregated and vesiculated.
4.5	0.1 M Phosphate	"	0.1 M Phosphate buffer	1% OsO ₄ 2 hr	Fair to poor
"	"	"	"	1% OsO ₄ 8 hr	Fair
"	"	"	"	1% OsO ₄ 16 hr	Fair to good
"	"	"	"	Roth's fixative 2 hr	Fair to poor
"	"	"	"	Roth's fixative 8 hr	Poor
"	"	"	"	Roth's fixative 16 hr	Poor
"	"	"	"	3% OsO ₄ 2 hr	Fair
"	"	"	"	3% OsO ₄ 8 hr	Poor
"	"	"	"	3% OsO ₄ 16 hr	Poor
"	"	"	0.1 M Phosphate buffer + 10% sucrose	"	Poor
"	"	"	0.1 M Phosphate buffer	"	Fair

(a) Roth's fixative: 1% OsO₄ + 1 mM CaCl₂ at pH 7.16 buffered with sodium acetate and sodium barbital both at 0.014 M [*J. Cell. Biol.* **34**, 47–59 (1967)].

after orbital flight and to compare them with the controls grown terrestrially under similar conditions.

PROGRESS REPORT

Vibration Tests

Prior to the Bios II shot in August, 1967, a number of vibration tests were run to determine the tolerance of amoebae to vibration frequencies during gravitational stresses up to 12 *g*. These stresses are char-

vors, when compared with the unstressed controls, have yet been found.

Fixation Testing

The best fixatives for ultrastructural studies of amoebae require OsO₄, but fumes from this compound are extremely toxic. Consequently, there was a possibility that OsO₄ fumes would escape from the space provided and kill all life in the Bios spacecraft. Exploratory electron microscopic work was, therefore, done on a considerable number of substitute fixative Compounds used which failed to produce good results were: Alcohol-formalin (10 ml of 37% formaldehyde

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in 90 ml 85% ethanol); 1% KMnO_4 ; and ethanol, 15%. Glutaraldehyde showed the most promise, and we investigated the effects of various preparations containing this fixative (Table 59). One of the best preparations was 3% glutaraldehyde in 0.1% phosphate buffer, with 1 mM CaCl_2 added. Fixation was for two days, the length of the Bios II orbital flight; the rinse was made in plain 0.1% phosphate buffer (pH 7.5), followed by post-fixation for 16 hr in 1% OsO_4 . The nuclear structures of amoebae fixed in this preparation compared favorably with those prepared in Roth's fixative (see Table 59), although the nuclear envelopes had some breaks. Also, the mitochondria were aggregated, and most of them contained a large vesicle. With the exception of the abnormal vesicle

within the mitochondrion, its fixation was quite acceptable.

CONCLUSION

Various preflight studies were completed on *P. carolinensis* for the Bios II shot. The information obtained was adequate, and the orbital flight of Bios II was completed. Some of the organisms were fixed during the flight under weightless conditions, while others were recovered alive. A number of the latter group were fixed after recovery, but other amoebae from this group are being cultured to obtain physiological data on the effects of weightlessness on their growth and reproduction. The fixed amoebae are now being studied with the light and electron microscopes.

Cellular and Somatic Effects

STUDIES OF THE CORNEAL EPITHELIUM OF MICE. I. CELL KINETICS DURING THE PERIOD OF A DAY

R. J. Michael Fry and Charlotte L. Weber

PURPOSE AND METHODS

Studies on the diurnal variation of the number of cells in mitosis and in DNA synthesis in the mammalian corneal epithelium have been reported.⁽¹⁻⁴⁾ These studies have used what might be termed static indices and have not included the determination of fluxes. Thus, it is not known in what way the movement of cells through the various phases of the cycle is altered. Our first measurements were made on the movement of cells from post-synthesis (G_2) to mitosis (M).

Colcemid (0.08% Solution, Ciba) was applied topically to the eyes of 100-day-old female CF $\times$ 1/Anl [Anl 66] mice at 12 noon, 4 P.M., 8 P.M., 12 midnight, 4 A.M., and 8 A.M. Two mice were sacrificed at hourly intervals for each four-hour period. Whole mount preparations were made from a central-punch sample and a peripheral strip. The number of blocked metaphases per hundred fields was determined. A field was the area covered by a 1 mm $\times$ 1 mm grid, using an oil immersion 100 $\times$ objective and optovar 1.25. At least 50 fields of both samples of the epithelium from both eyes were examined. In this way, a majority of the total cell population was sampled.

PROGRESS REPORT

The results from the determination of the flux of cells from G_2 into M during the six four-hour periods of the day are shown in Figure 159.

Between 2200 and 0700 the flux is low. The number of cells in metaphase, after one hour of collection in metaphase at 1000, is about fifteen times greater than at 0200 or 2200.

It is clear that while the corneal epithelium is a steady-state system over a period of days, it does not exhibit steady-state kinetics over the period of one day. The next stage of these investigations has been held up by difficulties in obtaining reliable data by using the double label technique, which is presumed to be the method of choice for determining the flux of cells both in and out of DNA synthesis.

FIG. 159.—The number of Colcemid blocked metaphases per hundred fields of the corneal epithelium during six four-hour periods. Colcemid was given one hour before the time of the first data point of each four-hour collection period. Each point is the average of the data obtained from two samples of two eyes of two mice.

CONCLUSIONS

When these fluxes are determined, we should be able to give a more complete description of the changes in the cell kinetics occurring within the day and to

determine whether there are differences in the stage sensitivities to the environmental and endogenous factors that determine diurnal rhythms.

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STUDIES OF THE CORNEAL EPITHELIUM. II. THE CELL CYCLE AND ITS COMPONENT PHASES

R. J. Michael-Fry, Charlotte L. Weber and Janet Newlin

PURPOSE AND METHODS

The corneal epithelium is a cell renewal system of relatively simple design in which a large fraction of the cell population can be sampled. The difficulties involved in cell kinetic studies with this tissue are the marked lability of its mitotic activity and the inconstancy of its proliferative rates during a 24-hour period. For a full description of the renewal kinetics it is necessary to determine the following characteristics of the cell population: (1) the ratio of proliferative to adult cells, (2) the cell turnover rate, (3) the cell life-span, (4) the cell cycle, and (5) the number of maturation divisions. This report covers (1) the estimation of the duration of the cell cycle and its component stages; (2) a preliminary study of the fraction of the basal layer of cells in cycle; and (3) the ratio of adult to proliferative cells.

CF $\times$ 1/Anl [Anl 66] female mice were used. In the study on the cell cycle, one group of mice was injected with 20 μCi $^3\text{HTdR}$ (360 mCi/mM) between 8:30

and 9:30 A.M., and was sacrificed at intervals up to 80 hr afterward. Another group of mice was injected at various and appropriate hours, and all were sacrificed at the same time, 9:00 A.M. Autoradiographs of squash preparations of the corneal epithelium were made,⁽¹⁾ and the percent of mitoses labeled was determined.

To a third group, $^3\text{HTdR}$ (200 $\mu\text{Ci}/\text{ml}$) was applied topically to the cornea every 6 hr for 72 hr. Two or three mice were sacrificed at 12, 24, 36, 48, 60 and 72 hr, and autoradiographs of sections were made. The percent of cells labeled in the basal layer, and in the total cell population, was determined. The ratio of the number of cells in the basal layer to the total cell population was determined on these sections.

PROGRESS REPORT

The results of the percent mitoses labeled, as a function of time, are shown in Figure 160. The percent of mitoses labeled reaches 100 at 6 hr, which indicates

FIG. 160.—Percent mitoses labeled as a function of time. $\times$, Data obtained from mice injected at 9:00 A.M. and sacrificed at various times; $\circ$, data obtained from mice sacrificed at 9:00 A.M. and injected at various times.

FIG. 161.—Percent of labeled cells in basal layer (X) and in total epithelium (O) as a function of time. $^3\text{HTdR}$ was applied at 6-hr intervals for 72 hr.

that the maximum post-synthesis (G_2) and mitosis (M) is about 6 hr. The duration of DNA synthesis (S), estimated from either the area under the first part of the curve, or the time elapsed between the 50% points on the ascending and descending limbs is 14–15 hr.

The small rise in percent mitoses labeled between 42 and 56 hr suggests that a small number of cells may have a cycle time of about 40 hr. The subsequent larger rise, starting at 68 hr and continuing up to the latest sacrifice interval, 92 hr, indicates that the majority of cells have an intermitotic time of at least 64–76 hr. The part of the curve between 68 and 92 hr is “smeared out,” indicating a considerable variation in cell cycle times in this tissue.

The duration of DNA synthesis obtained by this method does not agree with the estimate (8–10 hr)

obtained by determining the flux of cells from S into mitosis.⁽²⁾ The explanation for this discrepancy may be the variations in the rate of flow of cells through phases of the cycle, described in a separate report.⁽³⁾ The results obtained by the percent mitoses labeled are influenced not only by the duration of S , but also by the duration of the other phases. For example, if the duration of G_2 is increased, even cells which leave S at a normal rate will be delayed in entering mitosis. This delay will result in the first part of the curve being wider than normal.

The results of the multiple application of label are shown in Figure 161. When label is continuously available to these cells, the percent of basal layer cell labeled should increase until all the cells in cycle come into S . The curve should then plateau at time nearly equal to G_1 , plus M , plus G_2 . A plateau below

100% indicates that not all of the cells in the basal layer are in cycle. Our preliminary data suggest that there is a flattening of the curve for percent of basal layer cells labeled versus time at about 72 hr.

Until data from later time intervals are obtained, it is not possible to estimate the fraction of the population that is in cycle.

There is no evidence that either the total cell population or the number of basal layer cells alters markedly over a period of days. In other words, this tissue is a *steady-state renewal system*. The increase in the number of labeled cells in the basal layer should reflect the rate of entry of cells into DNA synthesis, because any increase in labeled cells by division will be balanced by the movement of labeled daughter cells into the more superficial layers. The results indicated that the increase in labeled cells in the basal layer between 12 and 48 hr could be treated as linear function of time. From the slope of the regression line ($0.85 \pm 0.13\%/hr$) fitted to the data and the initial labeling index of the basal layer (8.6 ± 1.4), it can be seen that the flux is consistent with about a 10-hr DNA synthesis period. The increase in the percent of the total population labeled is due to both the entry of cells into *S* and also to division of labeled cells. The slope for the total population labeled is less than that of the increase of basal

layer cells labeled, because it reflects the turnover rate of the total population and not the flux of cells in cycle. The percent of the total population in the basal layer was 38.6 ± 2.8 on a sample of 21 eyes. All the cells in the basal layer are considered proliferative, though not all the cells are necessarily in cycle. When the number of cells in cycle is determined, the size of the reserve proliferative population can be calculated.

CONCLUSIONS

The cell cycle of the corneal epithelial cells is long, mainly because of the long G_1 . The results obtained to date underline the problems involved in determining the duration of DNA synthesis in tissues with marked diurnal rhythms.

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THROMBI IN THE LEFT ATRIUM OF THE HEART OF MICE

R. J. Michael Fry and Eugenia M. Cooke

PURPOSE AND METHODS

We have reported on the incidence of thrombi in the heart of BALB/c mice.⁽¹⁾ It was found that sex, age, breeding history and genetic makeup were factors that influence the incidence of atrial thrombi, but we could not elucidate the etiology of this condition. Meier and Hoag,⁽²⁾ who confirmed most of our findings, suggested that this thrombosis was associated with a postpartum rebound in the level of prothrombin to above-normal levels; the prothrombin level having been depressed during pregnancy. Sichuk et al.⁽³⁾ found no correlation between the occurrence of thrombi in Syrian hamsters and platelet numbers, or with various clotting times. They did identify that thrombi occurred earlier in gonadectomized than in untreated males. We are seeking answers to the following questions: (1) Is the incidence of thrombi related to anemia? (2) Do repeated pregnancies result in anemia? (3) Are the strain differences in the in-

cidence of thrombi related to strain differences in anemia? Both Monsen⁽⁴⁾ and we found that thrombi occur in mice fed evaporated milk.

The diet was also suspected as a cause of anemia, though no data in support of this suspicion were collected. It was also thought that the anemia might be related to the incidence of thrombi. So our investigations have begun with a study of the hematological changes and the incidence of thrombi in mice receiving a milk diet. Male and female BALB/c/Anl and CF $\times$ 1/Anl mice, 70-80 days of age, were maintained on evaporated milk for 8 to 9 weeks. Blood samples were taken from the orbital sinus at 1, 2, 3, 4, 7, and 8 weeks after beginning treatment, and at sacrifice 61 days after the onset of the diet in both experimental and control groups. The red blood cell count, hemoglobin level and packed cell volume were determined by standard methods. At autopsy, the mice were examined for any abnormality and after a pe-

TABLE 60. INCIDENCE OF THROMBI AND HEMATOLOGICAL DATA FOR FEMALE BALB/c MICE MAINTAINED ON EVAPORATED MILK AND NORMAL DIET

Strain	Sex	No. of mice	Diet	No. of days on diet	Packed cell vol. (PCV), %		Hb, g/100 ml		RBC $\times 10^6$		Mean corpuscular hemoglobin conc. (MCHC), %		No. of mice with thrombi
					Mean	SD	Mean	SD	Mean	SD	Mean	SD	
BALB/c	F	6	Milk	0	47.2	1.2	15.4	0.8	8.3	0.3	32.7	2.0	—
		6		7	43.1	4.0	14.9	1.2	8.1	0.6	34.0	1.0	—
		6		14	35.3	1.0	11.5	0.7	8.1	0.4	32.7	1.5	—
		6		21	32.8	2.0	11.5	1.0	7.9	0.7	35.3	4.1	—
		6		28	28.9	4.0	9.1	1.2	7.1	0.9	31.7	1.6	—
		5		49	15.0	4.0	6.1	1.1	5.5	1.0	41.2	6.3	—
		5		56	11.4	3.0	3.9	0.9	4.4	0.7	33.9	4.5	—
		5		61	8.2	2.0	4.0	0.4	3.6	0.4	51.5	11.9	5
BALB/c	F	6	Normal	0	47.9	4.1	15.9	1.8	8.4	0.8	33.0	1.5	—
		6		7	46.3	1.6	15.9	0.6	8.3	0.7	34.3	1.4	—
		6		14	46.3	2.0	16.3	0.7	9.5	0.8	35.1	1.1	—
		6		21	46.9	1.0	16.5	1.6	9.6	0.2	35.2	3.3	—
		6		28	44.8	0.8	16.0	0.3	8.8	0.4	35.6	1.2	—
		6		49	45.0	1.1	17.4	0.5	11.9	0.9	38.6	1.7	—
		6		56	44.6	0.5	16.3	0.6	11.4	0.4	36.5	1.2	—
		6		61	45.2	0.5	17.4	0.7	11.0	0.9	38.6	1.6	0

TABLE 61. INCIDENCE OF THROMBI AND HEMATOLOGICAL DATA FOR MALE BALB/c MICE MAINTAINED ON EVAPORATED MILK AND NORMAL DIET

Strain	Sex	No. of mice	Diet	No. of days on diet	Packed cell vol. (PCV), %		Hb, g/100 ml		RBC $\times 10^6$		Mean corpuscular hemoglobin conc. (MCHC), %		No. of mice with thrombi
					Mean	SD	Mean	SD	Mean	SD	Mean	SD	
BALB/c	M	6	Milk	0	48.3	1.9	15.9	1.3	9.0	0.5	32.9	2.7	—
		6		7	44.1	3.1	15.0	1.2	8.3	0.7	34.1	1.3	—
		5		14	35.6	0.7	11.5	1.0	7.4	0.4	32.3	3.2	—
		5		21	33.8	2.0	11.5	1.2	7.4	0.7	34.1	2.7	—
		5		28	30.8	1.2	10.2	0.3	8.1	0.9	33.0	1.3	—
		5		49	12.9	1.2	5.8	0.6	5.0	0.3	45.0	5.0	—
		5		56	9.7	1.7	4.3	0.3	4.1	0.5	45.6	6.0	1
		3		61	7.0	1.8	3.1	1.0	3.6	0.3	43.6	5.1	1
BALB/c	M	5	Normal	0	49.4	1.5	17.0	1.4	9.8	6.3	34.4	2.2	—
		6		7	44.9	2.6	16.1	0.8	9.0	0.5	35.9	2.5	—
		6		14	44.3	1.3	14.7	1.0	8.7	0.4	33.1	2.6	—
		5		21	45.2	2.1	15.8	0.7	9.0	0.5	35.1	1.4	—
		6		28	45.3	1.8	15.8	0.6	9.4	0.6	34.9	0.6	—
		6		49	44.1	0.8	17.5	0.7	11.3	1.3	39.8	1.6	—
		5		56	43.4	2.6	16.2	1.2	10.8	0.4	37.4	2.9	—
		6		61	44.7	1.8	17.1	1.1	10.7	1.5	38.1	1.3	0

TABLE 62. INCIDENCE OF THROMBI AND HEMATOLOGICAL DATA FOR CF#1 MICE MAINTAINED ON EVAPORATED MILK AND NORMAL DIET

Strain	Sex	No. of mice	Diet	No. of days on diet	Packed cell vol. (PCV), %		Hb, g/100 ml		RBC $\times 10^6$		No. of mice with thrombi
CF#1	F	9	Milk	61	29.3	5.1	11.3	2.6	8.3	1.5	0
	M	5	Milk	61	30.1	3.5	11.3	1.3	7.7	1.1	0
	F	5	Normal		47.3	2.3	—	—	11.7	1.2	0
	M	5	Normal		47.6	1.4	—	—	8.7	0.8	0

riod of fixation the heart was reexamined and cut coronally so that its four chambers could be inspected for evidence of thrombi.

PROGRESS REPORT

Two male and one female BALB/c/Anl mice died about 57 days after beginning the milk diet. No deaths occurred in the CF $\times$ 1/Anl mice. Five of the 6 female and 2 of the 6 male BALB/c/Anl mice had thrombi in the left atrium at autopsy. No thrombi were found in the CF $\times$ 1/Anl mice.

The results of the changes in the various hematological parameters are shown in Tables 60-62. From these results, and from previous experiments, it is clear that the evaporated milk diet caused a high incidence of atrial thrombi in the BALB/c/Anl mice, but not in the CF $\times$ 1/Anl mice, at least not in the eight weeks they were treated. The diet did cause an anemia, which was more marked in the BALB/c/Anl mice, presumably because of the repeated blood sampling of this group. In the mice maintained on the standard diet (Wayne Lab-Blox), the blood sampling at the same intervals resulted in a rise in the hemoglobin level, and the red blood cell count was similar to the count typically obtained as a result of bleeding. The appearance of the red blood cells, and of some nucleated cells on the blood films, suggest a macrocytic hypochromic anemia, but the corpuscular volume and the hemoglobin concentration are not consistent with such an anemia. With milk as the sole source of nutrition, it is possible that the anemia is related to a

copper deficiency. Further investigation will be necessary to determine the exact nature of the deficiencies involved. Ball et al.⁽⁵⁾ found a high incidence of atrial thrombi in mice fed a diet low in protein and high in fat. They did not identify an anemia but did observe myocardial damage that they believed contributed to the thrombosis.

CONCLUSION

While the induction of left atrial thrombi and anemia both occur in the BALB/c/Anl mice maintained on evaporated milk, an unequivocal cause and effect relationship has not yet been established. Experiments are in progress to establish the effect of the number of pregnancies on the hematological parameters and to examine strain differences in the production of thrombi as a result of milk diet.

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CELL PROLIFERATION IN THE INTESTINAL EPITHELIUM OF PEROMYSCUS LEUCOPUS AFTER 1, 3, 10, AND 35 DAYS' EXPOSURE TO 56 R/DAY WHOLE-BODY ⁶⁰Co GAMMA IRRADIATION

Samuel Leshner, Janie Bauman, and George A. Sacher

Recent studies of the life shortening effect of long-protracted ⁶⁰Co gamma irradiation indicate that *Peromyscus leucopus*, the white footed deer mouse, is probably more sensitive to daily exposures than the inbred laboratory LAF₁ mouse.⁽¹⁾

Peromyscus leucopus is an interesting animal for long-term aging and low-dose continuous irradiation studies since its life expectancy in the laboratory is about five years (maximum), while that of the LAF₁ mouse is approximately 3½ years. It is of special interest because of its paradoxical sensitivity to continuous daily irradiation at high daily dose levels. This radiation sensitivity may have a cellular basis and could result from differences in rate of cell pro-

duction in critical organ systems, e.g., intestinal epithelium and bone marrow. Experiments were planned and are in progress to study (1) size of the proliferative population and (2) duration of the generation cycle in the intestinal crypts of 100-day-old unirradiated animals and in animals exposed to 56 R/day for 1, 3, 10, and 35 days (exposure starting at 100 days of age).

Tritiated thymidine (³HTdR), a DNA label, will be used to establish the size of the proliferative population on a whole crypt basis (using a crypt squash technique) in normal animals and to study effects of irradiation. Duration of the generation cycle will be estimated by the labeled mitosis method.⁽²⁾

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EFFECTS OF SINGLE-DOSE, WHOLE-BODY IRRADIATION ON CELL RENEWAL IN RODENT INTESTINE

Samuel Leshner and Janie Bauman

PURPOSE AND METHODS

When it was found that the recovery of the intestinal epithelium following high single-dose gamma- and X-ray exposures^(1, 2) involved (1) an accelerated cell generation cycle and (2) an increase in the number of cells in the proliferative compartment, a series of experiments was planned to obtain data that would lead to a characterization of the cellular events which take place during the time course of radiation recovery:

Phase I involves a study of damage, repair, and recovery of the intestinal epithelium following a series of whole-body ^{60}Co gamma-ray doses (75, 150, 300, 500, 600, 750, and 1000 R).

Phase II, concerns the effects of split doses separated by various time intervals.

Phase III will deal with partial body irradiations to the abdominal region.

In each phase, a study will be made of the effects of irradiation on (1) DNA synthesis (number of cells involved and rate of synthesis), (2) the movement of cells through the cell cycle and the duration of this cycle at various times after exposure, and (3) cell-cycle-phase (G_1 , S , G_2 , and M) sensitivity. These studies will utilize the crypt squash technique and high resolution autoradiography after the injection of animals with tritiated thymidine ($^3\text{HTdR}$) to obtain estimates of total cell number, labeled nuclei and mitotic figures per crypt. Estimates of the duration of the generation cycle will be made, using the labeled mitosis method. The DNA synthesis rate will be determined by using a radiochemical counting technique which utilizes a Tri-carb liquid scintillation spectrometer to measure the tritium content of the crypt cells (20 crypts/sample).

PROGRESS REPORT

Preliminary data suggest that there is a general pattern of damage, repair, and recovery in intestinal epithelium. (See Figure 162.)

Immediate Effects

Cells are retarded or completely blocked in G_2 , and the duration of the G_2 block increases in relation to the dose. The number of cells per crypt in S and the rate of DNA synthesis are rapidly reduced by irradiation. The extent of reduction increases as the dose is increased. The rapid reduction of labeled cells suggests a G_1 block.

Recovery

As cells begin to divide after the repair of the G_2 block, the severely damaged cells are eliminated. Eventually, the crypt is repopulated with cells capable of repeated division. The number of cells capable of division increases rapidly, while at the same time, the generation time is accelerated. The number of labeled nuclei per crypt and mitotic figures per crypt exceeds the control level; the total number of cells within the crypt exceeds the normal number; a new steady state is established; and the production of cells tends to return toward a normal level. Extent of change is dose-dependent.

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TIME AFTER IRRADIATION

FIG. 162.—Generalized effects of whole-body irradiation on the intestinal epithelium which forms the inner lining of the mouse small intestine. Immediately after irradiation there is a rapid reduction in the total number of cells in the crypt, which includes the dividing cells in the proliferative compartment (P-zone) and the differentiating or maturation compartment (M_c-zone). The number of cells remains low until most damaged cells are eliminated and replaced in the P-zone. The rate of cell production increases rapidly, and the number of cells in the P-zone overshoots control levels. When the crypt has been repopulated with viable cells, the size of the P-zone has a tendency to return to normal. At high exposure levels the entire crypt appears to be capable of synthesizing DNA and dividing (P-zone). At the same time the distribution of cells in G₁, S, G₂ and M is disturbed. The generation cycle is temporarily accelerated as shown by lower circles.

A FOOT-OPERATED CELL COUNTER

William J. Eisler Jr., R. J. Michael Fry, and Donald le Buis

PURPOSE AND METHODS

Cell indices from autoradiographs and histological sections, as well as differential white cell counts from blood smears, routinely must be done by "hand." During those determinations, both the fine adjustment and the movement of the stage of the microscope are manipulated continually. This is tedious and inefficient, as one must remove his hand to operate the typical laboratory counter. We report a simple solution to this problem here.

open" contacts of the cumulative register. The preset feature of this register allows the alarm to be set to any number up to 9,999.

CONCLUSION

This foot-operated cell counter has worked satisfactorily in our hands and has increased the speed at which indices were tabulated. Little difficulty was experienced by the operator in locating the correct

FIG. 163.—A foot-operated cell counter

PROGRESS REPORT

The apparatus, shown in Figure 163, has a foot board with four pressure-sensitive switches and an enclosure for the components and circuitry. The electronics are completely solid state and consist of a separate board for each channel. The power supply is common to all channels. Each switch position is designed to discharge a capacitor into a "one-shot" multivibrator. The appropriate register driver and the register respond to this circuit. The cumulative register and a channel register are driven simultaneously by a diode "or" gate as shown in Figure 164. An audible alarm is connected to the "normally-

FIG. 164.—Block diagram of the complete system

pedal. Two additional switches could be added to the foot board by placing the switches in an arc and adding a heel support. In most counts, two classes of cells

are frequent and the remaining classes are infrequent; therefore, the amount of foot movement required by the operator in making his record is not excessive.

ULTRASTRUCTURAL STUDIES OF THE VASCULAR ENDOTHELIUM IN THE IRRADIATED CHICK EMBRYO

S. Phyllis Stearner and Margaret H. Sanderson

PURPOSE AND METHODS

Exposure of the chicken and chick embryo to ionizing radiations can produce vascular changes that lead to circulatory failure and death within a few hours. These effects represent an early expression of radiation damage that has been described in terms of dose, dose rate, and time (both for continuous and for fractionated exposures).^(1, 2) Light microscope studies of the circulation in the extraembryonic membranes of the chick embryo revealed that this early circulatory failure results from progressive vascular deterioration and stasis.⁽³⁾ The fine structural alterations in the endothelium associated with this early circulatory degeneration is described in this report.

Three-day chick embryos *in ovo* were X-irradiated in a Lucite incubator maintained at 38°C. The embryos were fixed for electron microscopy 2 hr after exposure to 1200 R of 250 kVp X rays, delivered at 95 R/min. The living embryo and entire area vasculosa, X-irradiated or unirradiated, was attached to a filter-paper ring, dissected from the yolk, and sacrificed by submersion *in toto* in Karnovsky's double aldehyde fixative, pH 7.4, at 24°C. The entire specimen and filter-paper ring was fixed for 1–2 hr, washed overnight in cold phosphate buffer, pH 7.4, and post-osmicated with Millonig's 1% fixative, pH 7.4, at 4°C for 1 hr. The specimen was dehydrated with ethanol and propylene oxide, and infiltrated with Epon. The filter-paper ring was then removed, and the embryo and surrounding membrane were transferred on a paper spatula to a flat-bottomed polypropylene dish that contained Epon. Polymerization was carried out at 60°C for 48–72 hr. Regions selected from the area pellucida and from the caudal region of the body of the embryo were cut from the Epon with a dental saw, mounted on aluminum rods, and sectioned on a Cambridge ultramicrotome. Sections were stained with 2% aqueous solution of uranyl acetate followed by Venable's lead citrate stain, and viewed in an RCA-EMU3E electron microscope.

PROGRESS REPORT

In the 3-day unirradiated embryo, small and medium sized blood vessels in the area pellucida and within

the embryo were composed of a layer of endothelium and occasionally a pericyte. In addition, processes of adjacent mesenchyme cells were closely apposed to the endothelium so that the vessel wall appeared to consist of a double or triple layer of cells (Figure 165a). Venules and capillaries could not be distinguished, for there is little differentiation of the vascular system at this stage of development. Small masses of condensed material outside the endothelium represented the beginning formation of a basal lamina or basement membrane, although it was not continuous at this stage of development. Tight junctions usually consisted of a zonula occludens and a zonula adherens. Well-defined desmosomal structures were not seen between the endothelial cells (Figure 165b). Processes of the endothelial cells, occasionally with some interdigitation of the junction, sometimes overlapped for relatively long distances (Figure 165b). Others were connected over only 0.1 μ or less (Figure 165c). The endothelial cells contained pinocytotic vesicles, some rough endoplasmic reticulum, and a large number of free ribosomes. Other cell organelles included mitochondria, a typical Golgi complex and several inclusion bodies. The endothelium was frequently very thin, less than 0.1 μ in thickness, over extended areas. The diameter of the smaller blood vessels ranged from 7 to 10 μ , and the erythrocyte was approximately 7 μ in diameter. The vascular endothelium in the extraembryonic membrane appeared to be continuous. Discontinuities were rarely seen and were considered anomalies or artifacts. Pinocytotic vesicles, regularly seen in the normal endothelium, were usually few in number; in general there seemed to be fewer vesicles in this material than are usually seen in mammalian endothelium.

Exposure to 1200 R of 250 kV X rays results in hemoconcentration and damage to the microcirculation within 2 hr, and mortality exceeds 95% within 24 hr. The embryos examined in this study, sacrificed at 2 hr after exposure, showed several alterations in ultrastructure of the endothelium. Some endothelial cells showed a great increase in the electron density of the cytoplasm. Occasional finger-like or bifurcated projections, sometimes of rarified but more commonly of densely packed cytoplasmic organelles, were

FIG. 165.—Control endothelium. a, Cross section of extraembryonic membrane containing part of a capillary; b, endothelial cell and intercellular junction; basement membrane present, but incomplete; c, Short intercellular junction between endothelial cells. L, Lumen; EN, endothelium; ER, erythrocyte; M, mesenchyme cell; BM, basement membrane; J, intercellular junction.

FIG. 166.—Alterations in endothelium following X irradiation. a, Electron-dense cytoplasm with rarified and electron-dense projections from endothelial cell; basement membrane present, but incomplete; b, Rarified cytoplasm and intracellular edema in endothelium; erythrocyte adherent to cell membrane; c, Similar to b; intercellular junctions intact; d, Circulating cell protruding into extravascular space. L, Lumen; EN, endothelium; ER, erythrocyte; M, mesenchyme cell; BM, basement membrane; J, intercellular junction.

FIG. 167.—Cross section through caudal region of X irradiated embryo showing presence (arrows), and absence (X) of endothelial barrier. ER, Erythrocyte; EN, endothelium; L, lumen.

formed (Figure 166a). Localized intracellular edema, in which the rarified cytoplasm was pale-staining and nearly devoid of organelles, developed in other cells. Dilatation of the endoplasmic cysternae and some swelling of mitochondria were also present (Figures 166b and c). The plasma membranes and the intercellular junctions of these cells remained intact. Occasionally circulating cells were attached to the endothelial cell membrane adjacent to areas of localized edema (Figure 166b), or protruded out into the extravascular space through gaps between endothelial cells (Figure 166d). Moderate hemoconcentration and disruption or absence of parts of the endothelial barrier indicated more severe vascular damage (Figure 167). In such areas, circulating blood cells readily gained access to the subendothelial spaces. When vascular damage was more severe, blood cells intermingled with other necrotic cells, forming masses of debris in which no endothelium could be iden-

tified. The intercellular junctions in undamaged endothelium appeared to have maintained their integrity.

The presence of hemoconcentration and intracellular edema suggested that subendothelial edema might also develop, although it is difficult to quantitate because of the loose mesenchymal structure surrounding the endothelium. Functional injury to the endothelium may be estimated more accurately with tracer particles such as mercuric sulfide, colloidal gold or carbon.

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EFFECTS OF IONIZING RADIATIONS ON DURATION OF LIFE IN THE CHICKEN. PROGRESS REPORT

S. Phyllis Stearner and Emily J. B. Christian

PURPOSE AND METHODS

White leghorn cockerels,* irradiated at 3 to 4 days after hatching, are being maintained for duration of life in order to determine long-term radiation effects on survival time. No long-term study of chickens maintained under laboratory conditions is known; for this reason an important part of the investigation involves study of unirradiated control birds. The radiation exposures were either to 200 kV X rays or to ^{60}Co gamma rays. All X-ray exposures were delivered in 30 min; gamma-ray exposures were given in either 6-60 min or in 24 hr, in order to investigate the effect of exposure time on dose effectiveness. Radiation doses covered the 30-day lethal range. At 30 days after exposure, survivors were caged individually and are observed regularly. Chicks were entered into the experiment at different times over a period of more than 6 years, and at the time of this report all had been observed for at least 8 years.

PROGRESS REPORT

In the control group of 95 roosters, mortality reached 50% in about 5 years; after 8 years 20% were still surviving.

Of those gamma-irradiated birds alive at 30 days after exposure, 50% survived about 3 years. There appeared to be little correlation between dose and

survival time over this dose range, but doses protracted over a 24-hr period were somewhat less effective than when delivered over 60 min. In the group of 58 roosters exposed over a period of 60 min or less, only 2 survived more than 6 years. In the group exposed over a 24-hr period, 8 out of 59 survived more than 6 years. This is in spite of the fact that the mean delivered dose in groups exposed over 24 hr was larger than in groups that received short exposures. (The short and long exposures, however, were comparable in their effect on 30-day mortality.) We have also observed a lack of correlation between dose and growth effect for doses within this range. X-irradiated roosters received 1000 to 1200 R. In a group of 68 birds, mortality was unusually high during the first year. Between 1 and 5 years the survival curve paralleled that of the control, but mortality was high thereafter, and only the last decile survived more than 7 years.

CONCLUSIONS

On the basis of data available from groups exposed to radiation doses in the 30-day lethal range, we estimate that irradiation decreased survival time by 2 or 3 years. It should be emphasized that these data were obtained from a laboratory-reared group and may not be representative for chickens raised in large flocks. The mortality observed in the control group resembles that which has been reported for zoo populations.

* Obtained from Kerr Chickeries, Frenchtown, New Jersey.

PROTECTIVE EFFECT OF SOYBEAN TRYPSIN INHIBITOR AGAINST EARLY RADIATION MORTALITY

S. Phyllis Stearner and Steven A. Azuma

PURPOSE AND METHODS

The early radiation response in the chicken can be considered an inflammatory reaction, resulting in a physiological condition similar to irreversible shock. The early decrease in plasma volume and protein concentration suggests an increased vascular permeability.⁽¹⁾ Terminal events observed in the circulation of the irradiated chick embryo include hemoconcentration, capillary engorgement, and cell stasis, as well as degenerative changes in the vascular endothelium.⁽²⁾ Recent publications contain suggestions that radiations may affect intracellular membranes, resulting in increased permeability. For example, alterations in lysosomal membranes may be followed by release of hydrolases, such as proteolytic enzymes, into the cell and ultimately into the circulating blood. Little is known about the mode of action of these substances in relation to vascular permeability, but Jolles and Harrison⁽³⁾ have suggested that activation of such enzymes, or decrease of their inhibitors, is responsible for the early permeability response of irradiated rabbit skin. In addition, increase in plasma hydrolytic enzymes has been reported after induction of experimental shock.⁽⁴⁾

The protective effect of soybean trypsin inhibitor (SBTI), an antiprotease, on early radiation mortality in the 14-day chick embryo had been reported previously.⁽⁵⁾ The effect of SBTI on survival of the ⁶⁰Co gamma-irradiated chick is described here. Acid phosphatase concentration of the plasma was used as an indicator of the level of proteolytic activity; determinations were made in control and irradiated chicks and were compared with similar determinations obtained after administration of SBTI. Male white leghorn chicks, three days old, were exposed to ⁶⁰Co gamma rays. The exposure time was 10–12 min. Soybean trypsin inhibitor, in 0.1 ml of solution, was administered 60 min or 30 min before irradiation. Other chicks received either 0.1 ml of 0.7% saline or nothing. For determination of plasma acid phosphatase activity, blood was obtained by cardiac puncture of unanesthetized chicks. Tubes containing blood samples were placed immediately in ice. Plasma was separated by centrifugation at 1,000 rpm for 10 min at 4°C and was stored overnight at 5°C. Acid phosphomonoesterase activity was determined by the method described by Berger,⁽⁶⁾ with minor modifications. Aliquots of plasma (0.1 ml) were incubated with disodium *p*-nitrophenyl phosphate substrate in

FIG. 168.—Effects of soybean trypsin inhibitor (SBTI) on mortality within 24 hr after ⁶⁰Co gamma irradiation of the 3-day chick.

citrate buffer, pH 4.8, at 38°C for 30 min. Acid phosphatase activity is expressed as mM *p*-nitrophenol released at 38°C/hr/liter plasma.

PROGRESS REPORT

The protective action of SBTI, administered 30 min before irradiation, on early mortality (within 24 hr after exposure) in the 3-day-old chick is shown in Figure 168. The amount of protection increased with increase in dose up to 75 mg/kg. Larger amounts of SBTI provided less protection, and doses above 200 mg/kg produced deaths due to drug toxicity. SBTI administered 60 min before irradiation was less effective in reducing early mortality than when it was given 30 min before irradiation. The maximum reduction in radiation effectiveness observed in groups that received 75 mg/kg SBTI 30 min before irradiation was equivalent to about 800 R. In the chick embryo, effective doses of SBTI were similar to those in the chick, but the amount of dose reduction did not exceed 400 R.⁽⁵⁾

Plasma acid phosphatase activity in the 3-day-old chick averaged about 0.6 mM/1/hr (Table 63). This is similar to the activity reported by Bell and Campbell in 15- to 18-week-old chickens.⁽⁷⁾ Activity appeared to be slightly greater in chicks that received soybean trypsin inhibitor, but was not altered by saline alone. The effect of ⁶⁰Co gamma irradiation

TABLE 63. CHANGES IN PLASMA ACID PHOSPHATASE AFTER ^{60}Co GAMMA IRRADIATION

Treatment	Time after irradiation, ^(a) hr					
	1	2	3	4	6	8
Control	0.590 (35)					
Control + 0.1 ml saline	0.640 (7) ^(b)	0.480 (6)		0.474 (6)	0.450 (6)	
Control + SBTI, 50 mg/kg	0.790 (6)	0.710 (6)		0.620 (6)		
700 R		0.740 (8)		0.840 (14)	0.800 (14)	0.740 (14)
1200 R	0.750 (6)	1.433 (23)		1.164 (4)		
1200 R + 0.1 ml saline		1.080 (20)	1.468 (9)	1.300 (7)		
1200 R + SBTI, 50 mg/kg	0.764 (6)	0.764 (9)		0.764 (6)		

^(a) Saline or SBTI administered at -0.5 hr.^(b) Activity expressed in mM *p*-nitrophenol/l plasma/hr at 38°C. Numbers in parentheses indicate animal sampled in each group.

TABLE 64. RELATION BETWEEN PLASMA ACID PHOSPHATASE ACTIVITY AND SURVIVAL IN IRRADIATED CHICKS

Treatment	Time after irradiation, ^(a) hr					
	1	2	3	4	6	8
Control	0.590 (35)					
Control + 0.1 ml saline	0.640 (7) ^(b)	0.480 (6)		0.474 (6)	0.450 (6)	
Control + SBTI, 50 mg/kg	0.790 (6)	0.710 (6)		0.620 (6)		
1500 R + 0.1 ml saline	0.750 (8)	1.370 (14)	1.520 (6)			
1500 R + SBTI, 75 mg/kg ^(c)		0.804 (9)		0.860 (9)	0.924 (8)	
“ “ ^(d)		1.244 (6)	1.320 (6)			0.800 (4)

^(a) Saline or SBTI administered at -0.5 hr.^(b) Activity expressed in mM *p*-nitrophenol/l plasma/hr at 38°C. Numbers in parentheses indicate animals sampled in each group.^(c) No mortality within 24 hr after exposure.^(d) More than 50% mortality within 8 hr. Animals sampled at 8 hr would probably have survived more than 24 hr.

on enzyme activity is shown in Tables 63 and 64. There was little change within 8 hr after a minimal lethal dose (700 R), but after a lethal exposure (1200 R) values increased to more than twice that of controls (Table 63). Irradiated chicks that received protective quantities of SBTI, however, showed little increase in plasma acid phosphatase activity.

The relation between increased plasma acid phosphatase activity and mortality in irradiated chicks is perhaps best demonstrated by result obtained in groups exposed to 1500 R. Most chicks that had received SBTI, before they were exposed to this radiation dose, survived more than 24 hr (Figure 168). In one group, however, mortality was high (more than 50% in 6 hr) and enzyme activity was also elevated well above control levels (Table 64).

CONCLUSION

Soybean trypsin inhibitor, a crystalline globulin protein, is reported to inhibit serum PF (permeability actor) and kallikrein as well as trypsin and chymotrypsin. There are no reports, however, of its effect on other proteases. We have demonstrated that SBTI protects against early radiation mortality in the 3-

day-old chick as well as in the 14-day-old chick embryo. The suggestion that the mechanism of action is through inactivation of proteolytic enzymes is consistent with results of acid phosphatase measurements in irradiated and SBTI-treated chicks. A twofold increase in plasma acid phosphatase activity was demonstrated after a lethal radiation exposure (1200-1500 R of ^{60}Co gamma rays), but there was only a slight increase in enzyme concentration after a minimal lethal dose (700 R). Groups that received SBTI before a lethal exposure showed a similar minimal increase in plasma acid phosphatase. This enzyme was selected as a representative cellular acid hydrolase to reflect the level of proteolytic activity. Although the specific action of SBTI on acid phosphatase has not been determined, findings of decreased enzyme levels in SBTI-treated, irradiated chicks is also consistent with the suggested implication of acid hydrolases in the early radiation effects in the chick. It is probable, however, that the increase in plasma activity is a secondary effect that reflects release of excess protease from damaged cells. Little increase in plasma acid phosphatase was observed at one hour after a lethal irradiation. An early increase would be

expected if such a change were a significant factor in initiating the vascular degeneration that is observed within a few hours.

In this connection, the early radiation-induced radioresistance that develops within 4 to 6 hr after an initial nonlethal exposure is of special interest.⁽⁸⁾ Resistance of previously irradiated chicks to a subsequent larger exposure may result from temporary elimination of some subcellular factor (e.g., depletion of an intracellular source of proteases). Restoration of sensitivity toward the normal level after 12 to 24 hr may then reflect reconstitution of this factor.

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PROLIFERATIVE CAPACITY OF PIG KIDNEY CELL CLONES SEVERAL YEARS AFTER IRRADIATION

Agnes N. Stroud and Dorothy L. Resh

PURPOSE AND METHODS

We are investigating the fate of pig kidney cell clones derived from an irradiated culture.^(1, 2) The original culture was irradiated with a single dose of 500 R X rays, and on the basis of cell counts it was estimated that about 20% of the cells survived. Thirty hours after irradiation the mean chromosome aberration rate in the culture was two per cell; at two weeks, 0.62; and at one month, 0.15. At that time 14 clones were isolated, five of which survived after two months' subcultivation. All have consistently shown abnormally high aberration rates; one clone failed to survive at 30 months, and two died out after about three years. On the basis of this early death, or chromosome changes and alterations in growth rate and metabolic characteristics, it is assumed that all of the clones were genetically altered. At the same time, some recovery has been apparent in the longest survivors. While increases in aberration rates have been observed in cultures failing to survive, the original culture appeared to return to normal within a few months.

This report concerns the growth characteristics and radiosensitivity of the two surviving clones and the parent line.

PROGRESS REPORT

The pig kidney cell line maintained in our laboratory for seven years has a pseudodiploid chromosome

number of 37, of which one is a marker chromosome, formed at an early stage of subcultivation by the fusion at the centromeres of two of the largest acrocentric chromosomes (M). The karyotype of the parent line is shown in Figure 169A. Karyotypes of the two surviving clones; a few months after isolation and irradiation, are shown in Figure 169B and C.

Clone 3 had three new types of chromosomes, or marker chromosomes (Figure 169B). Clone 14 also had three radiation-induced marker chromosomes (Figure 169C), different from clone 3, and the parental marker M.

The most frequent type of radiation-induced chromosome markers found in the two clones during their proliferation after irradiation were reciprocal translocations and exchanges. The new markers were formed by the separation of rejoined chromosome breaks, or from breaks at radiation-induced gaps in the chromosome, involving sister chromatid exchanges or nonhomologous chromatid exchanges. While the clones showed changes in patterns of chromosome markers, with time, the latest markers have been demonstrated to be quite stable.

The types of radiation-induced markers now seen in clone 3 and clone 14 are shown in Figure 170. Clone 3 (Figure 170C) has only one marker (MU), which is a subtelocentric chromosome. It is larger than the subtelocentric chromosome GI and slightly smaller than the parent line marker M. The mean ratio of

FIG. 169.—Karyotypes of the pig kidney parent line and its clones 6 months after 500 R and isolation. A, Unirradiated parent line with the normal marker M; B, clone 3 with missing chromosomes (—) and M and three new radiation-induced markers (???); C, clone 14 with missing chromosomes (—), M, and three new radiation-induced markers (???).

FIG. 170.—The types of radiation-induced chromosome markers in clones of pig kidney cells 4 years after irradiation. A, Clone 14, GI and GIII are normal subtelocentric chromosomes, and MU-A is the radiation-induced marker; B, GVII and GVIII are normal acrocentric chromosomes and MU-B is the radiation-induced acrocentric marker; C, clone 3, M is the normal marker, MU is the radiation-induced marker, and GI is the normal subtelocentric chromosome.

the chromosome length of M/MU is 1:1.11. The mean ratio of the chromosome length MU/GI is 1:1.24. The mean ratio of the long arm to the short arm of MU is 1:2.4, of GI, 1:1.68, and of M, 1:1.0. This marker was found in 60% of the cell population and was present in cells with chromosome numbers of 37 and 38. The modal chromosome number of the clone was 38, and 26.5% of the cells are in this group (Table 65). Clone 14 has two radiation-induced chromosome markers, one subtelocentric and one acrocentric (Figure 170A and B). The subtelocentric marker (MU-A) falls in length between the largest subtelocentric chromosome GI and the second largest

subtelocentric chromosome, GIII. The mean ratio of the chromosome length of MU-A/GI is 1:1.34 and of GIII/MU-A, 1:1.25. The mean ratio of the short arm to the long arm of MU-A is 1:1.70 and of GI and GIII it is 1:2.06 and 1:2.67, respectively. The second marker (MU-B) is an acrocentric and is slightly smaller than the second largest acrocentric, GVII. The mean ratio of the chromosome length of GVIII/MU-B is 1:1.15. The telocentric marker MU-A was present alone in 57% of the cells and was present with the acrocentric marker MU-B in 35% of the population. The MU-B marker did not appear without an MU-A marker. Neither marker was present in 7.3% of the cells. Cells with 38 chromosomes contained the greatest number of both markers (MU-A and MU-B), or the MU-A marker alone. The modal chromosome number of this clone was 38. The modal chromosome number of the parent line was 37.

After irradiation there was an increase in doubling times in these clones, which have returned to normal at this time; the present doubling time of the irradiated clones (3 and 14), relative to the parent population (one day), is 1.03 and 1.05 days, respectively.

The response of the clones to further irradiation

TABLE 65. FREQUENCY DISTRIBUTION OF THE PERCENT OF CHROMOSOMES IN THE PARENT LINE AND IRRADIATED CLONES FOUR YEARS AFTER IRRADIATION

Cell line	Chromosome number								
	32	33	34	35	36	37	38	39	40
	Percent of cells								
Parent line	0	18.0	16.4	18.5	6.1	41.0	0	0	0
Clone 3	6.1	2.1	16.3	16.3	8.2	16.3	26.5	4.1	4.1
Clone 14	0	6.7	27.0	3.3	6.7	6.7	47.0	0	0

FIG. 171.—Surviving fractions of the parent line, clone 3, and clone 14 as a function of the X-ray dose, 6-days after irradiation (100 R/min).

four years after the initial dose was investigated by cell counts six days after irradiation and compared with the parental line after 50, 100, 200, 300, 400, 500, 700, 1000 R, X rays. The surviving fraction after these doses of irradiation is shown in Figure 171. The extrapolation number for the two clones (3 and 14), based on several experiments, is about 1.4. The D_{37} of the parent line is 159 R, and those of clones 3 and 14 are 135 R and 145 R, respectively. From these data it appears that the clones are no more sensitive than the parent line to a second dose of irradiation.

CONCLUSIONS

Although the clones now show no differences in growth rate from the parent line, when grown on the complete medium ordinarily used (Morgan's 199 and 10% lamb serum), they have genetically determined metabolic differences which are apparent when the clones are grown on a less complete medium lacking certain amino acids (Eagle's and 10% lamb serum without serine, glutamic acid, aspartic acid, alanine, proline, hydroxyproline, and glycine). On the incomplete medium it was found that their growth rates were significantly less than growth rates of the parent line, although they appear to have become adapted to full growth on the complete medium.

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EFFECTS OF HIBERNATION ON RADIATION SENSITIVITY

Bernard N. Jaroslow and Marilyn A. Williams

PURPOSE AND METHODS

Lethally X-irradiated (1100 R) ground squirrels do not die as long as they remain in hibernation (up to 3 months). When they are removed from hibernation, the mortality during the following 50 days is the same

as in the nonhibernating controls.⁽¹⁾ This suggests that hibernation does not alter radiation sensitivity, that it stops or slows down the development of radiation injury, and that recovery from radiation is not enhanced by extended hibernation. From studies of

the development of the immune response,⁽²⁾ it became apparent that radiosensitive processes can be carried on during hibernation. Therefore, in order to better investigate the rate of recovery, during hibernation, from radiation injury, ground squirrels were given fractionated doses of radiation.

The ground squirrels (*Citellus tridecemlineatus*) were captured, housed, and induced to hibernate in the manner described in earlier reports.⁽¹⁻³⁾ They were examined twice daily for deaths.

PROGRESS REPORT

In an initial experiment, it appeared that more recovery (as indicated by survival) occurred after 3 doses of 400 R of X rays, over a 2-week period, to ground squirrels hibernating at 5° C than after similar irradiation of ground squirrels kept at 23° C. A single dose of 1200 R to hibernating animals gave 85% mortality after 30 days at 23° C.⁽³⁾ In the succeeding years^(4, 5) the LD_{50/50} for a single exposure to ⁶⁰Co gamma radiation was established. The LD_{50/50} for the control ground squirrels was 1152 ± 11 R, whereas those that had spent 3 weeks in hibernation had an LD_{50/50} of 1266 ± 36 R.

To assess the amount of recovery between exposures better, the experiment was redesigned so that the ground squirrels were given two doses of 750 R of ⁶⁰Co gamma irradiation 6 hr apart. In this interval between exposures, 90% of the total recovery occurs in mice and chickens.^(6, 7) Animals were irradiated while one group was in hibernation and the other awake. The hibernating ground squirrels were returned to room temperature one day after irradiation and allowed to awaken. The mortality during the 50 days after irradiation was 6 of 18 in the group irradiated while hi-

bernating and 11 of 17 in the group irradiated while awake.

CONCLUSIONS

There are two likely explanations for these results. Either radiosensitivity is decreased by hibernation or recovery is greater during hibernation. The study is being continued to determine which of these factors is dominant.

We wish to thank Mr. S. Tyler for carrying out the statistical evaluation of the data.

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MODIFICATIONS OF ANTIBODY FORMATION IN RABBITS

William H. Taliaferro and Lucy G. Taliaferro

PURPOSE AND METHODS

The particular modifications of antibody formation in this study of rabbits involved widely different initial immunization procedures with the Forssman antigen in minced horse kidney (HK); heavy radia-

tion of rabbits with an exposure dose of 500 R two days before the start of immunization; and separation of the antisera by starch zone electrophoresis, sucrose gradient ultracentrifugation and 2-mercaptoethanol treatment.

FIG. 172.—The initial anti-Forssman hemolysin responses in rabbits 1 and 6 which were injected once with antigen and in rabbits 11 and 22 which were intensely immunized by repeated antigen injections (arrows). Rabbits 6 and 22 were irradiated with 500 R two days before the start of antigen injections. Note the more intense response of repeatedly injected rabbits 11 and 22 as compared to singly injected rabbits 1 and 6, and the more rapid appearance of hemolysin in unirradiated rabbits 1 and 11 as compared to irradiated rabbits 6 and 22.

PROGRESS REPORT

Figure 172 shows representative hemolysin responses in heavily irradiated rabbits and unirradiated controls, injected only once or 20 times in 23 days with the antigen. On the one hand, the initial hemolysin response in irradiated rabbit 22 during intense immunization reached as high peak titer as in unirradiated rabbit 11, which was similarly immunized, although the latent period before the appearance of hemolysin was prolonged. On the other hand, the initial hemolysin response elicited by a single injection of antigen in irradiated rabbit 6 was markedly depressed and delayed as compared to similarly immunized, unirradiated rabbit 1. When these rabbits were reinjected with HK four months later, the anamnestic response showed a typically faster reactivity than during the initial response.

The declines in the initial responses after peak titer in rabbits 11 and 22 were characteristic of the groups to which they belonged, and the temporary early decline and delay in reaching peak titer in the response in rabbit 11 was encountered in a few of the rabbits. These declines in titer represent specific unresponsiveness in marked antibody excess during continued antigenic stimulation, as reported by us in 1962. The antigenic stimulation, possibly because of the insoluble nature of the antigen, was inadequate to lead to

specific unresponsiveness in marked antigen excess during continued antigenic stimulation, as has been reported by others for some soluble antigens.

The anti-Forssman sera collected from the foregoing series were analyzed for their immunoglobulin content with respect to the relatively large, electrophoretically fast-moving IgM type and the small, slow-moving IgG type. The results from four of them are shown in Figure 173. During the initial response in rabbits intensely immunized with antigen (Figure 173 A and C), over 95% of the hemolysin produced was of the IgM or γ_1 type; the remainder was of the IgG or γ_2 type. This same ratio was found in the singly injected series and in the irradiated intensely immunized series. During the anamnestic response (Figure 173 B and D), however, the IgG type increased slightly (up to 15%) and occasionally predominated in rabbits that had received several series of repeated injections of antigen.

The X-ray sensitivity of hemolysin formation of both immunoglobulins is clearly shown by the delayed appearance of hemolysin in rabbits 6 and 22 in Figure 172. The high titer in irradiated rabbit 22 in Figure 172 is not considered to indicate a radio-resistance, but is rather related to the efficacy of continued antigenic stimulation during recovery of the rabbit from the injury inflicted by irradiation.

FIG. 173.—Hemolysin and protein determinations in antisera from intensely immunized rabbits after separation by zone electrophoresis (A and B) and sucrose gradient ultracentrifugation (C and D). Serums were from initial (A and C) and anamnestic (B and D) responses. SP, the starting point in A and B. Note the essential absence of IgG hemolysin in A and C and the appearance of a small amount of it in B and D.

CONCLUSION

Under the conditions of these experiments, the relative amounts of the two anti-Forssman immunoglobu-

lins produced are not primarily dependent upon the amount of the Forssman antigen injected but rather on the reactivity of the rabbit. Formation of both types is radiosensitive.

THE INDUCTION OF ANTIBODY SYNTHESIS

Bernard N. Jaroslow and Beverly A. Serrell

PURPOSE AND METHODS

The induction of the primary and the secondary antibody response is studied in hibernating ground squirrels (*Citellus tridecemlineatus*). The development of the response can be slowed by permitting the animals to hibernate. As demonstrated in earlier studies,⁽¹⁻³⁾ the latent period (the interval between the injection of antigen and the appearance of antibodies) can be lengthened from the 7 days in nonhibernating animals to 56 days in hibernating ground squirrels.

EFFECTS OF HIBERNATION ON THE DEVELOPMENT OF THE SECONDARY RESPONSE

While the anamnestic response has been described in detail, the developmental stages are poorly known. In this series of experiments, the secondary response was initiated at different times from 32 to 103 day after the primary immunization. Ground squirrels were permitted to hibernate during the interval between injections. The stay in hibernation ranged from 14 to 56 days.

TABLE 66. EFFECTS OF HIBERNATION ON THE SECONDARY RESPONSE

Group	Days ^(a) Hib.	Days post-hib. rec. ^(a)	Days between injections ^(a)	Primary response			Secondary response			<i>p</i> ^(b)
				Mean Peak Titer			Mean Peak Titer			
				Log ± S.E.	Geo-metric mean	No.	Log ± S.E.	Geo-metric mean	No.	
Experiment 1										
1C	0	—	32	3.04 ± 0.07	1100	10	3.19 ± 0.08	1550	10	>0.05
1H	14	0	32	3.02 ± 0.11	1050	8	2.96 ± 0.03	914	8	>0.05
2C	0	—	45	2.86 ± 0.07	725	9	3.39 ± 0.10	2460	9	<0.001
2H	28	0	45	2.55 ± 0.08	355	10	2.74 ± 0.07	550	10	>0.05
3C	0	—	74	3.00 ± 0.08	1000	9	3.32 ± 0.09	2090	8	<0.02
3H	56	0	74	3.09 ± 0.06	1220	10	2.81 ± 0.10	645	9	>0.05
Experiment 2										
4C	0	—	—	2.36 ± 0.08	230	6	—	—	—	—
4H	28	3	—	2.31 ± 0.11	204	7	—	—	—	—
5C	0	—	48	2.87 ± 0.07	740	7	3.60 ± 0.07	3980	8	<0.01
5H	28	3	48	2.78 ± 0.18	600	8	2.82 ± 0.19	660	10	>0.05
6C	0	—	58	2.36 ± 0.08	230	10	3.37 ± 0.08	2340	9	<0.001
6H	28	14	58	2.27 ± 0.11	186	9	2.77 ± 0.11	590	9	<0.002
7C	0	—	76	2.28 ± 0.11	190	10	3.21 ± 0.08	1620	10	<0.001
7H	28	28	76	2.28 ± 0.12	190	10	3.15 ± 0.08	1410	8	<0.001
8C	0	—	103	2.18 ± 0.08	151	8	3.21 ± 0.09	1620	8	<0.001
8H	56	28	103	2.21 ± 0.10	162	8	3.05 ± 0.10	1120	8	<0.001
Experiment 3										
9C	0	—	45	2.14 ± 0.06	138	9	2.97 ± 0.13	934	10	<0.001
9H	28	3	73	2.09 ± 0.08	123	10	3.12 ± 0.11	1320	8	<0.001

^(a) These three columns show the days in hibernation, the number of days of posthibernation recovery before antigen injection and the number of days between the primary and secondary injections.

^(b) These values of *p* indicate the probability, determined by Student's *t* test, of the similarity between the primary mean log peak titer, and the secondary mean log peak titer in each group.

The collection and the housing of ground squirrels, and the technique in inducing them to hibernate are described in detail by Jaroslow and Smith.⁽¹⁻³⁾ The animals were immunized with 1.6×10^8 sheep red cells, given intraperitoneally 3 times in one day. Serum samples were collected by bleeding from the orbital sinus seven times during the first two weeks after the initial series of antigen injections, and six times during the eight days after the second series of antigen infections. Each experimental, hibernating group (H) of squirrels was paired with a nonhibernating group (C).

The animals of the H groups were placed in the cold room between the 16th and 42nd day after the primary injection. The sole exception was group 4H (Table 66), whose hibernation took place before the

primary injection. After removal of the H animals from hibernation, both the H and C animals in each group (except group 4) were given a second series of injections.

The serum was titrated for hemolysin content, according to the procedure described in detail by Taliaferro and Taliaferro.^(4, 5) The latent period and the peak titer were determined, and the statistical probabilities for the differences between the primary and secondary response were obtained by the Student *t* test.

Progress Report

Experiment 1 was planned to determine (1) the interval between injections that would give a good secondary response, and (2) the effect of different pe-

FIG. 174.—All ground squirrels were immunized while in hibernation. Animals in hibernation, broken line; awake animals solid line.

riods of hibernation on the secondary response initiated upon arousal.

The mean peak titer in the secondary response was significantly higher than that of the primary response, when the interval between infections was 45 ($p < 0.001$) and 74 ($p < 0.02$) days (Table 66, groups 2C and 3C), whereas at an interval of 32 days the secondary response was not significantly higher ($p > 0.05$, Table 66, group 1C). The three groups of ground squirrels that were placed in hibernation for 14 (group 1H), 28 (2H), and 56 (3H) days, respectively, were given a second injection on the day of arousal. None of the three hibernating groups had a peak titer in the secondary response that was higher than that observed in the primary response ($p > 0.05$).

Thus, an interval of 45 or 74, but not of 32 days between primary and secondary injections, resulted in a higher mean peak titer of the secondary response. If the ground squirrels were in hibernation for 28 or 56 days of the 45- or 74-day intervals, respectively,

the peak titer of the secondary response was not significantly higher than that of the primary response.

Experiment 2 was designed to determine whether the inhibition of the secondary response after hibernation was permanent or temporary. Once again, the secondary response gave a higher peak titer for intervals between injections of 48 days or more (Table 66, groups 5C–8C). In the animals that hibernated, the secondary response was inhibited to varying degrees, if it was initiated at 2 weeks or less after arousal (Table 66, groups 5H and 6H). The secondary response was not inhibited, however, if it was initiated 28 days after arousal (Table 66, group 7H). When the hibernation period was doubled (56 days), initiation of the secondary response 28 days after arousal did not impair the ability of the animals to respond (Table 66, 8H). The latent period of the secondary response was characteristically shorter than that of the primary response, in both the hibernating and nonhibernating ground squirrels.

There was no detectable inhibition of the primary response initiated three days after arousal (Table 66,

FIG. 175.—All ground squirrels were immunized on the day of arousal from 56 days of hibernation. The animals were *not* permitted to hibernate after immunization.

group 4H vs. 4C). This is in full agreement with the results of earlier investigations.^(6, 7)

The third experiment was aimed at learning whether the poor secondary response observed in some of the earlier groups⁽⁸⁾ was due to immunization shortly after arousal or whether a minimum time at normal temperatures was required before the anamnestic response matured. Those squirrels injected 3 days after arousal, but which had been awake for 42 days before hibernation, produced a distinctly higher peak titer ($p < 0.001$) than they had in the primary response (Table 66, groups 9H and 9C).

Conclusions

These results demonstrate that a minimum interval between immunizations is necessary before the anamnestic response can be initiated. This response, as compared to the initial response, is characterized by a shorter latent period and a higher peak titer. When the interval between injections is 32 days, the re-

FIG. 176.—All ground squirrels were allowed to hibernate 2 days after immunization, i.e., during the latent period. Animals maintained in hibernation, broken line; awake animals, solid line.

FIG. 177.—All ground squirrels were allowed to hibernate 4 days after immunization, i.e., during the period of logarithmic increase of antibody-forming cells. Animals maintained in hibernation, broken line; awake animals, solid line.

sponse is typical of a primary response. When the interval is 45 days or longer, the response is an anamnestic response. When a period of hibernation is interposed between the two injections, the amount of antibody produced after the second injection is no higher than the first, if the animal has not been awake for 45 of the days between injections. On the other hand, the short latent period is typical of the secondary response if the interval between injections, regardless of the stay in hibernation, is 45 days or more.

The cellular events of the latent period, in which hibernation inhibits the intensity of the antibody response without inhibiting the maturation of the synthetic mechanism, will be investigated.

EFFECTS OF HIBERNATION ON THE DEVELOPMENT OF THE PRIMARY RESPONSE

Jaroslow and Smith⁽¹⁻³⁾ found that the events of the latent period, up to the verge of the antibody synthesis, can take place during hibernation in animals immunized during hibernation. Titration of serum for

FIG. 178.—All ground squirrels were kept awake after immunization.

antibody was negative during hibernation in animals immunized just before they entered into hibernation, but upon arousal, these animals started producing antibody.^(6, 7)

We investigated the response to antigen in ground squirrels allowed to hibernate: during immunization, at two days after immunization, and at four days after immunization. They were immunized by a single intraperitoneal injection of 1.6×10^9 sheep red cells. The immune response was measured by the Jerne Plaque Technique,⁽⁹⁾ in which the number of antibody-forming cells in the spleen are counted.

Progress Report

In the squirrels that were immunized in hibernation, the number of plaque-forming cells increased very slowly during the 56 days of hibernation (Figure 174). Upon arousal, the number of antibody-forming cells increased at an accelerated rate during the next 8 days. The number of cells at 8 days after arousal was about the same as the number for parallel control ground squirrels (Figure 175) that had been put into hibernation at the same time, but that were not immunized until the 56th day of hibernation, when the animals were aroused.

In the group which was put into hibernation after induction was complete, but before the end of the latent period, i.e., two days after immunization, the number of plaque-forming cells accumulated in peak numbers during hibernation. By the time of arousal, 56 days after antigen injection, the number of plaque-forming cells in the spleen was in decline (Figure 176).

When the ground squirrels were put into hibernation during the period of logarithmic increase in the number of antibody-forming cells, i.e., 4 days after immunization, the peak number, the rate of increase and the subsequent decline of the number of plaque-forming cells (Figure 177), were similar to those values for the parallel nonhibernating controls that were immunized at the same time (Figure 178). The resurgence in the number of plaque-forming cells after arousal (Figure 177) may indicate the existence of a population of cells stimulated during hibernation.

Conclusions

The early part of the latent period is slowed by hibernation. As the immune response progresses, the number of antibody-forming cells increases rapidly in hibernation. The decline of plaque-forming cells in hibernators that starts 6 days after antigen injection is as rapid as in the nonhibernating ground squirrels. The work will be continued to investigate the kinetics of antibody synthesis and the dynamics of the cellular processes during the hibernation.

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THE SEPARATION OF ANTIBODY-FORMING CELLS BY SUCROSE DENSITY GRADIENT

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PURPOSE AND METHODS

Lymphocytes from fowl blood have been fractionated by equilibrium centrifugation in a bovine albumin density gradient.⁽¹⁾ Large, medium, and small lymphocytes were found throughout the gradient, showing an independent but overlapping density distribution.

By using the gradient differential centrifugation method, attempts were made to fractionate the spleen lymphocytes of mice into different classes according

modified model of Bock's gradient-former. Linear sucrose gradients of 10 to 40%, with a total volume of 14 ml, are used in all experiments. One milliliter spleen-cell suspension was carefully layered on top of the gradient. Centrifugations were performed in the swinging bucket (rotor #253) of the International Centrifuge Model PR-2 at 1,000 rpm ($250 \times g$) for 15 min. Eleven successive fractions were carefully isolated with a syringe and a specially bent needle, proceeding from the top to the bottom of the gradient

FIG. 179.—The distribution of nucleated cells (O) and plaque counts (X) from a 10 to 40% linear sucrose gradient. The gradient was centrifuged at 1,000 rpm for 15 min.

to their size. The centrifugations done in the present study are not in density equilibrium; the cells are thus separated according to their sedimentation rate.

CF#1/Anl [Anl 66] mice (20 g) were given an intravenous injection of 1.6×10^8 sheep red blood cells and were splenectomized 3 days later. A suspension of spleen cells was obtained by teasing the spleen in 3 ml of Eagle's minimum essential medium. This suspension was then centrifuged at a low speed in a clinical centrifuge for 90 sec, and the pellet of red blood cells in the bottom of the tube was discarded.

The sucrose gradients were prepared from a

tubes. Because of the greater cellular density in the top half of the gradient, smaller fractions were collected at the top half and larger ones at the bottom half of the gradient, with the following different volumes in the various fractions: fraction 1, 0.7 ml; fractions 2 to 4, 0.5 ml; fractions 5 to 8, 1 ml; and fractions 9 to 11, 1.5 ml; the remaining material was considered as the bottom layer.

Each fraction was tested for plaque-forming cells, according to the technique of Jerne et al.⁽²⁾ The number of nucleated cells in each fraction, as well as in the original cell suspension, were counted in a hemocytometer.

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PROGRESS REPORT

Sixty percent of the nucleated cells from the original cell suspension were recovered after the first low-speed centrifugation. The recovery of the plaque-forming cells was rather low, and varied between 12 to 20% in all experiments except one in which it was 80%.

The plaque-forming cells were distributed along the gradient in two distinct groups; the first group was found in the top 3 or 4 fractions, and the second in the last 3 or 4 fractions (fractions 9 to 11, see Figure 179A). The intermediate fractions 5 to 8 had very few plaque-forming cells.

The white cells (nucleated) were mainly distributed into two populations: one a population of small cells with a peak at fraction 4 and the other a population of larger cells with either one single peak at fraction 9 (Figure 179A) or two peaks at fractions 9 and 11 (Figure 179B). The peak at fraction 11 corresponded closely to that of the second peak of plaque-forming cells (Figure 179B).

CONCLUSIONS

1. The sucrose density gradient centrifugation method is feasible for the separation of white blood cells.

2. These results show the presence of two discrete populations of plaque-forming cells which have different sedimentation rates.
3. The cause of a low recovery in the plaque-forming cells in these experiments is, however, unknown.
4. The plaque-forming capability demonstrated by the cells might merely be the result of a leakage of antibody from the cellular membrane; hence, this is not necessarily an expression of cellular viability.
5. Whether the second peak of plaque-forming cells is constituted solely of large size cells or is contaminated by clumps of small cells and cellular debris is unknown.

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Laboratory Animal Medicine

DISEASE CONTROL STUDIES

Robert J. Flynn, Thomas E. Fritz, Calvin M. Poole, Ronald W. Camden, Patricia C. Brennan, and David V. Tolle

PURPOSE AND METHODS

New methods of controlling or eliminating disease in the animals used in the Division's research programs are continually being developed, and methods previously developed are continually being reassessed.

The procedures currently in use are quite complex. They have been briefly described in previous reports⁽¹⁻³⁾ and have been discussed in detail in a recent publication.⁽⁴⁾

PROGRESS REPORT

During the period covered by this report, a computer system was developed to record, analyze, and present in a useful manner the results of all periodic tests of our several rodent colonies. It is expected that this system described elsewhere in this report⁽⁵⁾ will materially assist in monitoring the health status of the animals used in the Division's research program.

Continuing periodic analyses of the productivity of our breeding colonies appears to be an effective way of recognizing possible disease problems. During the past year a decrease in fertility and neonatal viability in the dog colony was noted. This was investigated and the results are described in detail in the section on Toxicity and Metabolism of Radionuclides.^(6, 7)

Routine necropsy of all spontaneous and experimental decedents also appears to be an effective way of recognizing possible disease problems. This is illustrated by the high incidence of thyroiditis and the other spontaneous lesions noted in the dog colony. These, too, are described in the section on Toxicity and Metabolism of Radionuclides.^(8, 9)

During the past year, 7,310 water bottles from the rodent breeding rooms were cultured for *Pseudomonas aeruginosa*, and 7,071 fecal samples were cultured for

TABLE 67. RESULTS OF VIRUS SURVEY OF SOME ANL-BIM RAISED RODENTS

Strain	Number tested	Number with significant titers									
		Pneumonia virus of mice	Reovirus type 3	Theiler's mouse encephalomyelitis (GD VIII)	K virus	Polyoma	Rat virus	H-1 virus	Mouse adeno-virus	Mouse hepatitis virus	Sendai
SD/Anl[Anl 66]	11	0	0	0	0	0	0	0	0	0	4
CF#1/Anl[Anl 66]	24	0	5	0	0	0	(a)	(a)	0	12	0
CP/Anl[Anl 66]	21	0	2	2	0	0	(a)	(a)	0	4	0
CN-1/Anl[Anl 66]	19	0	4	1	0	0	(a)	(a)	0	3	0
A/Jax/Anl[Anl 66]	2	0	0	0	0	0	(a)	(a)	0	1	0
DBA/Anl[Anl 66]	8	0	0	0	0	0	(a)	(a)	0	2	0
C57BL/6/Anl[Anl 66]	3	0	1	1	0	0	(a)	(a)	0	1	0
HR/Anl[Anl 66]	5	0	0	0	0	0	(a)	(a)	0	1	0

(a) Not tested.

Salmonella and atypical *Citrobacter freundii*. Thirty-four cages were found infected with *P. aeruginosa*, 38 with *Salmonella* (*S. newington*) and none with *C. freundii*. No evidence of clinical disease was observed. Disposal of infected animals and maintenance of a high level of sanitation appear to be effective methods of controlling these infections.

The results of serologic tests of the rodent breeding colonies for latent viruses during the past year are given in Table 67. These findings indicate that the present procedures are effective in maintaining the colonies relatively free of viruses.⁽¹⁰⁾

CONCLUSIONS

The present disease control procedures appear effective. As a result of these procedures, several previously unrecognized possible disease problems were noted during the past year. Most have been defined, and all are under control.

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MURINE PNEUMONIA

Patricia C. Brennan, Thomas E. Fritz, and Robert J. Flynn

PURPOSE AND METHODS

In our last report⁽¹⁾ we suggested that murine pneumonia might be the result of synergism between *Pasteurella pneumotropica* and *Mycoplasma pulmonis*. It also seemed possible that *M. pulmonis* is an L form of *P. pneumotropica*. The latter hypothesis was

studied by the GC base ratios (molar ratio of guanine + cytosine/adenine + thymine + guanine + cytosine $\times$ 100) and nucleic acid homology techniques. The pathogenicity of these organisms was studied using axenic mice.

Deoxyribonucleic acid (DNA) was extracted⁽²⁾

TABLE 68. PROPERTIES OF THE DNA FROM *Pasteurella pneumotropica* AND *Mycoplasma pulmonis*

DNA source	Ratio of absorbance		S20w	Mol. wt. ($\times 10^6$)	Tm, °C	Moles % GC $\pm$ S.E.
	230/260 μ	280/260 μ				
<i>P. pneumotropica</i>	0.451	0.539	26.2	17.5	88.2 88.1 88.3	46.1 $\pm$ 0.15
<i>M. pulmonis</i>	0.408	0.535	17.5	5.2	82.4 82.1 82.8 82.6	32.1 $\pm$ 0.6

TABLE 69. MOLECULAR HYBRIDIZATION BETWEEN *P. pneumotropica* DNA AND *M. pulmonis* DNA

Recipient DNA attached to filter	³ H donor DNA ^(a)	% ³ H DNA bound $\pm$ S.E.	Relative % bound
<i>P. pneumotropica</i>	<i>P. pneumotropica</i>	32.6 $\pm$ 0.8	100
<i>P. pneumotropica</i>	<i>M. pulmonis</i>	3.0 $\pm$ 0.7	6.7
<i>M. pulmonis</i>	<i>M. pulmonis</i>	41.7 $\pm$ 3.8	100
<i>M. pulmonis</i>	<i>P. pneumotropica</i>	3.9 $\pm$ 0.3	5.2

^(a) The ratio of recipient to donor DNA was 20:1.

from both organisms. Radioactive labeling of each organism was accomplished by adding 5 mCi ³H-thymidine/liter of broth. The thermal denaturation temperature (Tm) was determined⁽³⁾ and used to calculate the per cent molar GC content. Sedimentation coefficients were determined in a Spinco Model E ultracentrifuge at 33,450 rpm using ultraviolet optics. Molecular hybridization between the two organisms was determined on nitrocellulose membrane filters.⁽⁴⁾

Three groups of female axenic CD-1 mice, 21–28 days of age, were obtained from the Charles River Breeding Laboratories. They were inoculated as shown below. Each group contained 50 animals which were housed in a plastic film isolator.

PROGRESS REPORT

The following experiments have been performed:

Experiment I. 2.5×10^7 colony forming units (CFU) *M. pulmonis*/mouse intranasally (I.N.).

Experiment II. 4×10^7 viable cells *P. pneumotropica*/mouse I.N.

Experiment III. 7×10^4 CFU *M. pulmonis* + 5×10^5 viable cells *P. pneumotropica*/mouse I.N.

Groups of 20 mice, inoculated with 0.05 ml sterile broth/mouse I.N., served as controls for each experi-

ment. Each group was housed in a separate isolator. Ten control animals were killed at the beginning, and 10 were killed at the end of each experiment. The lungs of all mice were cultured for microorganisms.

At 2 and 3 days after inoculation, and at 5-day intervals thereafter, 6 mice were removed from the chamber, killed by cervical dislocation, and necropsied. The lungs were removed, and portions were cultured for *P. pneumotropica* and *M. pulmonis*. Portions of 3 lungs were fixed in formalin for histopathologic examination. Frozen tissue sections from the remaining 3 lungs were prepared for fluorescent antibody studies and histopathologic examination.

Table 68 shows the properties of the DNA extracted from *P. pneumotropica* and *M. pulmonis*. The wide disparity in GC ratios suggests that there is no genetic relationship between the two organisms. Other investigators^(5,6) have pointed out that a disparity of more than 10% in the GC content is evidence that there are few pairs of nucleotides in common. This evidence is confirmed by the lack of duplex formation between the two DNA's (Table 69) although each is capable of duplex formation in the homologous system.

CONCLUSIONS

While analysis of the data from the *M. pulmonis* and *P. pneumotropica* pathogenicity studies is not complete, a few preliminary observations can be made. Both *M. pulmonis* and *P. pneumotropica* produce visible lung lesions in monoinfected mice. The lesions produced by an infection with both organisms are characterized by dark red areas of consolidation. In addition, abscess formation is a common finding in mice infected with *P. pneumotropica* alone. The lesions produced in mice simultaneously infected with *P. pneumotropica* and *M. pulmonis* more closely resemble those seen in naturally occurring murine pneumonia than do those observed in mono infected mice. These data, however, do not rule out the possibility that *M. pulmonis* is an L form of some other bacterial species whose GC content is closer to that of the mycoplasma. *M. pulmonis* is not an L form of *P. pneumotropica*.

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DISEASE STATUS OF COMMERCIALY PRODUCED RABBITS

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PURPOSE AND METHODS

Commercially produced rabbits are used extensively in research. However, the production of these animals has not been subjected to the rigid environmental and quality controls frequently used in the production of mice and rats. Personal communications with a number of scientists in other laboratories indicate that disease problems in *purchased* rabbits are

Prior to euthanasia they were examined for external evidence of disease or abnormality. They were then killed and examined for parasites and internal signs of disease. Nasopharyngeal cultures were made.

PROGRESS REPORT

All 10 vendors supplied the correct sex and number of animals. Only 5 of the 10 vendors provided the

TABLE 70. DISEASES AND PARASITES OBSERVED IN COMMERCIALY PRODUCED RABBITS

Vendors	Breed	Incidence of disease signs and parasites						
		Diarrhea	Respiratory disease	Ear mites	Tapeworm cysts	Coccidia	Intestinal nematodes	<i>Pasteurella multocida</i>
A	New Zealand white	1/10 ^(a)	0/10	0/10	6/10	9/10	3/10	6/10
B	New Zealand white	0/10	0/10	0/10	3/10	9/10	0/10	0/10
C	New Zealand white	0/10	0/10	0/10	2/10	10/10	5/10	4/10
D	New Zealand white	1/10	0/10	3/10	1/10	2/10	9/10	2/10
E	New Zealand white	0/10	0/10	2/10	0/10	0/10	0/10	5/10
F	Dutch belt	0/10	0/10	7/10	2/10	0/10	1/10	2/10
G	Dutch belt	2/10	0/10	3/10	0/10	7/10	10/10	0/10
H	New Zealand white	0/10	0/10	2/10	0/10	0/10	0/10	5/10
I	Dutch belt	0/10	0/10	1/10	0/10	4/10	2/10	0/10
J	New Zealand white	1/10	4/10	5/10	0/10	9/10	3/10	3/10

^(a) Number affected/number examined.

a very common occurrence. The disease incidence in the rabbits used in this laboratory has been increasing and has lead to the question of whether the diseases were acquired before or after procurement.

To determine if animals free of the common diseases are available, sample random-bred New Zealand white and Dutch belt rabbits were purchased from 10 commercial sources listed in *Animals for Research*.⁽¹⁾ Vendors were selected on the basis of geographic location to obtain nationwide representation.

Ten animals, 5 male and 5 female, in a weight range commonly used in this laboratory, were ordered, and their birth date was requested. The weight specified for the New Zealand whites was 5½ to 6½ pounds; for the Dutch belts, 3½ to 4½ pounds. The rabbits were isolated on arrival and killed within 48 hr. On receipt, they were compared with the order specifications as to number, sex, weight, and birth date.

birth date. No vendor provided all animals within the specified weight range. The number of animals per shipment which deviated from the weight specifications ranged from 1 out of 10 to 6 out of 10.

The results of this disease survey are summarized in Table 70. Antemortem examination of animals from 6 of the 10 vendors disclosed no signs of disease. Animals from 3 sources exhibited diarrhea only. Another group showed diarrhea in 1 animal, and signs of respiratory disease ranging from nasal exudate to rales in 4 animals. In addition, 2 animals in this group had severe dental malocclusion.

The only external parasites observed were ear mites, which were found in 1 or more animals from each of 7 of the 10 vendors.

The internal parasites observed included coccidia, intestinal nematodes and intraabdominal tapeworm cysts. In animals from 5 of the 10 vendors, tapeworm

cysts were demonstrated. Animals from 7 groups had intestinal nematodes, and 7 groups had coccidia. Only 2 groups did not harbor internal parasites, yet both of these had ear mites.

The nasopharyngeal cultures revealed a wide variety of bacterial organisms, the significance of which has not yet been determined. However, *Pasteurella multocida*, the organism reported to cause "snuffles" in rabbits, was identified in animals from 7 of the groups.

CONCLUSIONS

The majority of the commercial rabbit suppliers provide animals which carry several parasites, as

well as *Pasteurella multocida*. In addition, it was concluded that the vendors are not completely reliable as suppliers of animals as specified. The high incidence of such easily controlled parasites as ear mites and tapeworm cysts leads one to question the total husbandry and management practices that permit them to exist.

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THE NATURAL OCCURRENCE OF EXENCEPHALIA IN CF#1 MICE

Robert J. Flynn

PURPOSE AND METHODS

Exencephalia in the CF#1 mouse has been noted after X irradiation of gravid females, and a cause

and effect relationship has been suggested.^(1, 2) In animals with exencephalia, part of the brain protrudes and forms a cap on top of the head. This protrusion

FIG. 180.—Exencephalia in a 20-day fetus (right); normal fetus (left) included for comparison.

FIG. 181.—Typical appearance of a uterus containing a number of 20-day fetuses, one of which is exencephalic. The placenta with the exencephalic fetus is the one containing the blood-tinged fluid (arrow).

is not covered by bone or skin. Exencephalia has been reported in the mouse,⁽³⁻⁶⁾ but there are no reports of its natural occurrence in the CF#1 strain (Figures 180-181).

Defective offspring in rodents may not be viable when born, may die before being observed, or may be eaten by their mother at or soon after parturition.⁽²⁾ Thus, to determine the existence of teratogenic defects in rodents one should examine the fetuses at or just before parturition; to determine the natural incidence in a strain, one should examine several hundred fetuses.

To initiate disease-free breeding colonies, hundreds of CF#1 female mice were delivered of their offspring by cesarean section between the 14th and 20th day of gestation. Exencephalia was seen frequently. To determine its more exact incidence, a count was made of its occurrence during a two-week period.

PROGRESS REPORT

During the two-week period, 90 cesarean deliveries were performed, and 1056 fetuses were examined. Eleven litters contained exencephalic fetuses, one per litter. Thus, the percentage of the litters affected (11 of 90) is 12.2%, and the percentage of the fetuses affected (11 of 1056) is 1.04%.

CONCLUSIONS

The incidence reported is that which occurred in the ANL-BIM animal facilities in the absence of any known teratogenic agents. Thus, it is reported as the "natural" incidence. This incidence is specifically applicable to these facilities under the usual environmental conditions existing in them at the time of the study. It may not be applicable to other facilities with different environmental factors.

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PHYSIOLOGIC REGULATION OF BLOOD PLATELET PRODUCTION

David V. Tolle, Thomas E. Fritz, and Robert J. Flynn

A humoral poietin or "factor" which would reduce the degree of radiation-induced thrombocytopenia when given after exposure to near mid-lethal doses would be valuable in the treatment of radiation hemorrhage.

The therapeutic value of platelet transfusion in controlling the hemorrhagic phase of radiation illness is reported in the literature.^(1, 2) Unfortunately, the effectiveness of such treatment depends on the use of fresh, intact, viable platelets which will circulate in the recipient. Storage of whole blood or platelet concentrates by methods employed at present alters platelets so they will not circulate when transfused.

PURPOSE AND METHODS

Recent studies of physiologic regulation of hematopoiesis suggest that the regulation of all cellular elements of the blood is controlled by specific humoral factors similar to erythropoietin, which controls erythrocyte production.^(3, 4) Although there is evidence to indicate the existence of a circulating "Thrombopoietic Stimulating Factor" (TSF),^(5, 6) its structure, occurrence and its specific site of action (e.g., bone marrow, spleen, etc.) have not been clearly established.

The purpose of our studies is threefold: (1) to determine if TSF can be increased in mice in response to hematologic stress in which platelet levels remain normal, (2) to determine if mice, made thrombocytopenic by X irradiation, are useful in assaying for this factor and (3) to determine if TSF, when given after exposure to radiation, will reduce the degree of thrombocytopenia which develops. Because TSF activity has been associated with anemia in many species (rabbits, rats, and man),⁽⁷⁾ an attempt was made to produce such activity in mice by injecting them with phenylhydrazine hydrochloride, a chemical known to produce anemia in man and animals by destroying the erythrocytes;⁽⁸⁾ with other tissues (bone marrow, etc.) a secondary response may be involved.

Five daily intraperitoneal injections of 0.1 ml of 1.0% phenylhydrazine hydrochloride in physiological saline were given to 300 CF $\frac{1}{2}$ /Anl [Anl 66] female mice, 6-8 weeks of age. Platelet counts and bone marrow examinations were performed on 5 mice killed each day for 5 days. On the 6th day, the remaining 275 mice were killed by exsanguination, and this blood was collected in EDTA.* Platelet counts were performed on 22 animals. Plasma was obtained by removing the cells by centrifugation at 10,000 g. Normal plasma was similarly obtained from 275 untreated mice. The plasmas were stored at -90°C.

In another group, 40 untreated CF $\frac{1}{2}$ /Anl [Anl 66] female mice were used to determine the mean platelet count.

Three hundred mice, divided into three groups of 100 each, were used to assay the plasmas for TSF. The first group was given 600 R plus 0.1 ml of plasma from the phenylhydrazine hydrochloride-treated mice intraperitoneally each day for 10 days; the second group was given 600 R plus 0.1 ml of normal plasma intraperitoneally each day for 10 days; the third group was given only 600 R.

Platelet counts were made, using phase microscopy, according to the method of Brecher and Cronkite,⁽⁹⁾ and necropsy examinations for gross pathology were done on 10 mice killed from each group, daily, for 10 days. Wright-Giemsa-stained smears of peripheral blood and bone marrow from 2 of the 10 killed animals were also examined.

PROGRESS REPORT

The mice given phenylhydrazine hydrochloride showed a severe anemia by the 5th day of treatment. The mean hemoglobin value was 3.1 g/100 ml of blood and the packed cell volume was 20.6%. Although there was little change in the bone marrow at 6 days, peripheral blood smears showed marked toxic

* Dipotassium ethylenediamine tetraacetate (Sequester-Sol), Cambridge Chemical Products, Inc.

and degenerative changes by the 3rd day of treatment. Metarubricytes (nucleated red cells—last stage before loss of the nucleus) were observed in the peripheral blood smears on days 4 and 5. The mean platelet count was 785,858/mm³ for the 22 treated mice and 757,100 for the 40 untreated mice.

On days 5 through 10, the platelet counts of the irradiated group given normal plasma were significantly higher than for the irradiated control group, and the counts of those given plasma from the phenylhydrazine-treated animals were significantly higher [$p = < 0.05\%$ —Student's t test] than those given normal plasma. Comparisons of bone marrow morphology showed that erythropoietic recovery, as measured by erythrocytic cellularity, occurred earlier in plasma recipients than in the controls. The increased platelet counts in mice receiving plasma from phenylhydrazine-treated animals was accompanied by increased megakaryocyte recovery in the bone marrow.

The only gross changes seen at necropsy were splenic nodules in all groups, but these nodules were seen in greater number and size in animals given both types of plasma than in control animals. Such nodules are usually considered to represent the repopulation of the spleen by colonies of hematopoietic cells surviving irradiation.⁽¹⁰⁾

CONCLUSIONS

Plasma from normal mice appears to contain TSF activity; plasma from mice made anemic with phenylhydrazine hydrochloride appears to contain even greater TSF activity.

Mice made thrombocytopenic by X irradiation are useful as a model system to assay animals for TSF activity.

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THE DEVELOPMENT OF COLONIES OF DEFINED ANIMALS

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PURPOSE AND METHODS

The Division's research programs require more highly controlled and defined animals than are available from commercial breeders. In some cases, the strains needed are not commercially produced. For these reasons, several breeding colonies were initiated to assure the Division's research staff a supply of high quality, dependable animals. These now include 26 rodent colonies, of which 15 are cesarean derived or specific pathogen free (SPF), and an inbred and an outbred dog colony.

A monogamous, planned random-mating system is used in all rodent colonies, except in five inbred mouse colonies which are maintained with sibling matings.

A computer program is utilized for analysis and management of the breeding data.^(1, 2) Weanlings are selected for size, and those not within the desired weight range are discarded.

The cesarean derived rodent colonies are continuously monitored microbiologically. All are free of atypical *Citrobacter freundii*, *Mycoplasma*, *Pasteurella pneumotropica*, and both internal and external parasites. An occasional cage positive for *Salmonella* or *Pseudomonas aeruginosa* has been identified. The mouse colonies have no evidence of PVM, K, polyoma, Sendai, and mouse adenoviruses, but some significant titers for reovirus Type 3, GDVII, and mouse hepatitis viruses have been observed. The rat colony

TABLE 71. RECOMMENDED STRAIN DESIGNATIONS FOR RODENTS PRODUCED BY ANL-BIM^(a)

Strain designation	Common name
SD/Anl [Anl 66]	SPF Sprague-Dawley rat
CF #1/Anl [Anl 66]	SPF CF #1 mouse
CP/Anl [Anl 66]	SPF Catalase positive mouse
CN-1/Anl [Anl 66]	SPF Catalase negative M-1 mouse
A/JaxAnl [Anl 66]	SPF A/Jax mouse
DBA/1Anl [Anl 66]	SPF DBA mouse
BALB/cAnl [Anl 66]	SPF Bagg albino mouse
C3Hf/Anl [Anl 66]	SPF C3Hf mouse
C57BL/6Anl [Anl 66]	SPF C57 black mouse
Mastomys/Anl [Anl 66]	SPF Multimammate mouse
HR/Anl [Anl 66]	SPF Hairless mouse
RH/Anl [Anl 66]	SPF High radiation sensitive mouse
RL/Anl [Anl 66]	SPF Low radiation sensitive mouse
RC/Anl [Anl 66]	SPF Control radiation sensitive mouse
B6CF ₁ /Anl [Anl 66]	SPF BCF ₁ hybrid mouse
CF #1/Anl	Conventional CF #1 mouse
CN-2/Anl	Conventional catalase negative M-2 mouse
CN-3/Anl	Conventional catalase negative M-3 mouse
CN-4/Anl	Conventional catalase negative M-4 mouse
CN-6/Anl	Conventional catalase negative M-6 mouse
CHa/Anl	Conventional Chinese hamster
PD4/Anl	Conventional PD4 albino Syrian hamster
DEA/Anl	Conventional dark eared albino Syrian hamster
Crm/Anl	Conventional cream Syrian hamster
Gd/Anl	Conventional golden Syrian hamster

^(a) Based on References 5 and 6.

demonstrates no evidence of any of the above viruses nor either rat or H-1 viruses. The conventional rodent and the dog colonies are also continually monitored, and their pathogen status is known.

PROGRESS REPORT

Most of the cesarean derived rodent colonies developed or reinitiated during the period covered by

the last report⁽³⁾ are now in full production. No evidence of clinical disease was observed during this period.

Additional productivity data have been accumulated and stored by the computer program.⁽²⁾ These stored data have been used to analyze various facets of rodent productivity. A report of these analyses is given elsewhere in this section.⁽⁴⁾

New strain designations, based on the system suggested by Staats and Snell^(5, 6) were assigned to the Division's germfree and germ-low (SPF) rodent colonies. These designations, along with the common name of the strain, are listed in Table 71. The change involves the addition of brackets which enclose the abbreviation of the person or institution initiating a germfree or germ-low state, and the year it was done.

The rodent breeding colonies produced approximately 65,000 mice, 25,000 rats, and 1,800 hamsters for the Division's use during the period covered by this report.

The dog breeding colonies produced 162 animals during the year. Detailed descriptions of the status of these colonies are given elsewhere in this report.^(7, 8)

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STUDIES OF RODENT PRODUCTIVITY

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PURPOSE AND METHODS

Little is known of the effects of physiologic and environmental factors on productivity of rodents.^(1, 2) For this reason we have developed a computer program to analyze our stored data on tens of thousands of litters of rats and mice,^(3, 4) some of which were obtained under varied physiologic and environmental conditions. The first analyses have been concerned with the effects on productivity of parity, age of the female at mating, level of light, and environmental temperature.

The effect of parity on productivity was determined by calculating the mean numbers born and weaned per litter for each parity up to ten for the CF $\times$ 1/Anl

TABLE 72. EFFECT OF PARITY ON PRODUCTIVITY IN THE CF $\times$ 1/Anl [Anl 63] MOUSE

Parity	Born		Weaned		Percent weaned of those born
	Number of litters	Mean number per litter	Number of litters	Mean number per litter	
1	1976	9.6	1576	8.4	69.9
2	1456	10.7	1269	9.5	77.3
3	1015	10.9	923	9.6	80.0
4	674	11.0	604	9.5	77.3
5	412	10.6	341	9.3	72.6
6	234	10.3	192	9.1	72.4
7	130	9.7	94	8.9	66.3
8	66	7.9	45	7.2	62.1
9	27	7.1	19	7.0	69.3
10	7	6.1	5	5.8	67.9

TABLE 73. EFFECT OF PARITY ON PRODUCTIVITY IN THE SD/Anl [Anl 64] RAT

Parity	Born		Weaned		Percent weaned of those born
	Number of litters	Mean number per litter	Number of litters	Mean number per litter	
1	620	11.0	554	10.3	83.6
2	534	11.4	494	10.9	88.4
3	482	10.5	435	10.1	86.8
4	412	10.3	346	9.5	77.4
5	317	9.4	269	8.9	80.3
6	239	9.0	195	8.7	78.8
7	158	8.3	120	8.0	73.2
8	101	7.1	77	7.0	75.1
9	54	6.1	43	6.4	83.5
10	30	6.9	22	6.7	71.2

TABLE 74. EFFECT OF PARITY ON PRODUCTIVITY IN THE CF $\times$ 1/Anl [Anl 58] MOUSE AND SD/Anl [Anl 63] RAT

Parity	CF 1/Anl [Anl 58] mouse		SD/Anl [Anl 63] rat	
	Number of litters	Mean number per litter	Number of litters	Mean number per litter
1	2412	9.5	313	11.5
2	2220	10.4	305	11.8
3	1987	10.3	299	10.6
4	1681	10.1	266	9.4
5	1321	10.0	243	9.4
6	992	9.8	226	8.8
7	692	9.1	197	8.1
8	463	8.4	146	7.9
9	305	7.7	91	6.5
10	186	6.8	52	5.9

[Anl 63] mice and SD/Anl [Anl 64] rats, and the mean numbers born per litter for the CF $\times$ 1/Anl [Anl 58] mice and SD/Anl [Anl 63] rats.

The effect of age of the female at time of mating was determined by dividing the data for all matings in the CF $\times$ 1/Anl [Anl 58] colony into four groups according to the age of the female at the time placed with a male of comparable age (30 days and less, 31–60 days, 61–90 days, and 91 days and above) and by calculating the mean numbers born and weaned per litter for matings within each group.

To study the effect of light, we measured the amount, in foot-candles, received at the front of each cage in a CF $\times$ 1/Anl [Anl 58] breeding room. The cages were then divided into three groups according to the amount received (0–30, 31–60, and 61–90 fc) and the numbers born and weaned per litter calculated for each group.

The effect of temperature was studied by changing the temperature in one CF $\times$ 1/Anl [Anl 58] mouse breeding room to 80° while leaving a comparable room at 73°. The average number born and weaned per litter was calculated for each environment.

PROGRESS REPORT

The effects of parity on productivity are shown in Tables 72 to 74. In all cases, the mean number born for parity one is less than that for parity two. The mean number born reaches a high at parity four in the CF $\times$ 1/Anl [Anl 63] colony and at parity two in the CF $\times$ 1/Anl [Anl 58] colony and in both rat colo-

TABLE 75. EFFECT OF AGE OF FEMALE AT TIME OF MATING ON LITTER SIZE IN THE CF #1/Anl [Anl 58] MOUSE

Age of female, days	Number of matings	Mean number born per litter	Mean number weaned per litter
<30	786	10.2	8.8
31-60	1502	9.9	8.1
61-90	216	9.8	7.9
>90	597	8.4	6.5

TABLE 76. EFFECT OF LIGHT LEVEL ON LITTER SIZE IN THE CF #1/Anl [Anl 58] MOUSE

Light level, fc	Number of litters born	Mean number born per litter	Mean number weaned per litter
0-30	2056	10.4	8.8
31-60	636	10.6	9.1
61-90	464	10.2	8.5

TABLE 77. EFFECT OF TEMPERATURE ON LITTER SIZE IN THE CF #1/Anl [Anl 58] MOUSE

Room temperature, °F	Number of litters born	Mean number born per litter	Mean number weaned per litter
73	2990	10.4	8.9
80	1543	9.3	8.4

nies. In the mouse colonies, the mean number born did not fall below the parity one value until parities seven or eight, while in the rat colonies this occurred at parity three. The mean number weaned per litter and the percent weaned of those born follows the same pattern. The number of pairs reaching succeeding parities decreases owing to natural attrition and culling.

MEDROXYPROGESTERONE ACETATE TO DELAY PARTURITION IN GRAVID RATS AND THE RESULTING EFFECT ON REPRODUCTIVE ABILITY IN CESAREAN-DERIVED PROGENY

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PURPOSE AND METHODS

Disease-free animal colonies can be initiated by foster nursing cesarean-derived neonates on germfree mothers. Proper timing of the cesarean section is necessary for optimum survival of the neonates. It is often necessary to delay parturition to achieve this timing.

The productivity of females mated at different ages is shown in Table 75. It is the highest in those mated at the earliest age and decreases for those mated later. Only litter size, and not number per mating, has been studied to date.

Tables 76 and 77 show the productivity at different levels of light and two different room temperatures. Again, only litter size, and not number per mating, has been studied to date. The largest litters born and weaned were at the intermediate light level, 31-60 fc, and at the lower of the two temperatures, 73° F.

CONCLUSIONS

Total productivity decreases with each succeeding parity with the possible exception of one and two. The rate of decrease is greater between later parities.

The optimum age for placing the female mouse with a male in at least one strain appears to be less than 30 days.

An intermediate level of light, 31-60 fc, appears to induce larger litters in mice than either higher or lower levels.

Litter size in at least one strain of mouse is greater at an environmental temperature of 73° F than at 80° F.

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We reported previously⁽¹⁾ that a single subcutaneous injection of 5.0 mg medroxyprogesterone acetate, given at 15½ days of gestation, will delay parturition in gravid mice. This treatment, however decreased the reproductive ability of the female progeny of treated dams. A single injection of the same drug in various dosages (0.5, 1.0, or 5.0 mg), given

at 17½ days of gestation, was equally effective in delaying parturition; none of the dosages used interfered with the reproductive ability of the female progeny of the treated dams. We have demonstrated, therefore, a time-dependence factor related to the period of gestation at which the drug is administered.

Because proper timing of cesarean sections in rats is as necessary for optimum survival of neonates as in mice, the treatment used successfully in mice was tried in rats. SD/Anl [Anl 66] female rats were exposed to males of the same strain once each week, for a 24-hr period. Conception was assumed to have occurred at the midpoint of this period. Some gravid females were allowed to deliver normally, serving both as controls against the parturition-delaying effects of the drug and as foster mothers for cesarean derived young. The remaining gravid females were given medroxyprogesterone acetate subcutaneously at 19½ days of gestation in dosages of 1.0 mg and 5.0 mg. Cesarean sections were performed at various intervals from 22½ to 25 days of gestation. The viability of neonates from all groups was noted as was the development of the external genitalia.

A study was also made of the reproductive ability of progeny of treated dams, and specifically the female progeny. Males and females within each group of offspring were mated to compare the reproductive ability of progeny of treated dams to that of progeny of untreated dams. Female progeny of treated and untreated dams were also mated with males from our production colony.

PROGRESS REPORT

The length of gestation for most of the control females (86%) was 22½ days or less (Table 78). If the drug delayed parturition to 22½ days, or later, and if cesareans were performed at that time, most of the neonates would be at or past the normal end of gestation. The 1.0-mg dosage was not completely

TABLE 78. LENGTH OF GESTATION OF SD/Anl [Anl 66] RATS

Treatment group	Number of females giving birth at designated time, days					
	21½	22	22½	23	23½	≥24
Control	5	33	25	3	7	0
1.0 mg medroxyprogesterone acetate at 19½ days	0	2	1 7 ^(a)	0	0	0
5.0 mg medroxyprogesterone acetate at 19½ days	0	0	57 ^(a)	0	16 ^(a)	2 18 ^(a)

^(a) Delivered by cesarean section; all others by normal labor.

successful in delaying parturition, but the 5.0-mg dosage was (Table 78). Cesarean sections were done between 22½ and 25 days on all the females given 5.0 mg, except for two females which delivered normally at 25 days. Two other females died at 25 days. There was nearly 100% viability in neonates derived surgically at 22½ days. In neonates taken at 23½ days, viability decreased; those taken at 24 days demonstrated an even greater decrease, and none were delivered alive after 24 days. No abnormalities of the external genitalia were noted in either the female or the male neonates. The types of matings made for the reproductive studies and the results of these matings are shown in Table 79. Two matings in each group, except the last, failed to produce a first litter. This failure is probably not significant, since two of these matings were between normally-derived animals from untreated dams. The proportion of matings having subsequent litters and the average interval between litters are similar for all groups.

TABLE 79. RESULTS OF MATINGS MADE TO DETERMINE REPRODUCTIVE ABILITY OF PROGENY OF SD/Anl [Anl 66] DAMS GIVEN 5.0 MG MEDROXYPROGESTERONE ACETATE AT 19½ DAYS' GESTATION

Type of mating	Total pairs observed	Number of litters and average interval between litters								
		1st	2nd	Days ^(a)	3rd	Days ^(b)	4th	Days ^(b)	5th	Days ^(b)
Delayed ^(c) male × delayed female	30	28	27	32	27	37	16	32	10	24
Normal ^(d) male × normal female	10	8	8	33	8	28	7	37	5	25
Breeding ^(e) male × delayed female	30	28	25	38	23	36	16	31	6	26
Breeding male × normal female	10	10	10	37	10	33	7	34	2	26

^(a) Average interval between 1st and 2nd litters.

^(b) Average interval between subsequent litters.

^(c) Delayed = cesarean-derived progeny from treated dams.

^(d) Normal = normally-derived progeny from untreated dams.

^(e) Breeding = animals from a production colony.

CONCLUSIONS

A single subcutaneous injection of 5.0 mg medroxy-progesterone acetate, given at 19½ days of gestation, satisfactorily delays parturition in the SD/Anl [Anl 66] rat. This dosage, given at this time, has no detrimental effect on the reproductive ability of progeny of treated dams.

AGE OF LABORATORY MICE AT PUBERTY

Ronald W. Camden, Ronald J. Svoboda, Robert J. Flynn, and Calvin M. Poole

PURPOSE AND METHODS

Since our observations on the age of laboratory mice at puberty were not compatible with the age given in the literature, studies were initiated in some of our strains to assess the accuracy of the literature and to determine the earliest age at which conception may occur in the female mouse, and the earliest age at which the male mouse is capable of inducing conception.⁽¹⁾ Last year, we reported that female CF#1/Anl [Anl 66] mice are capable of conception at 26 days of age and that the males are capable of inducing conception as early as 40 days of age. Observations of CN-1/Anl and CP/Anl females indicated that they are capable of conceiving at 20 and 19 days, respectively.

The studies of the CF#1/Anl [Anl 66] male were continued. Additional 21- or 22-day-old males were placed with adult females (60 days or older). This provided a practical test situation because the adult

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females were cycling continuously and could conceive when the male became old enough to induce conception.

Males and females of the CP/Anl [Anl 66], CN-1/Anl [Anl 66], and C57BL/6Anl [Anl 66] strains were also studied. Young (21-day-old) males of each strain were placed with adult females of the same strain and young (21-day-old) females with adult males. The 21-day-old males were in the same test situation as the CF#1/Anl [Anl 66] males above. The 21-day-old females were with males that were capable of inducing conception when the female became old enough to conceive. The age of an individual animal at conception or when inducing conception was calculated by subtracting the mean length of the gestation period (19 days)⁽²⁾ from the age of the experimental animals when the first litter was born. Adult female × adult male matings were also made within each strain to assure that animals of the age used were reliable test animals.

TABLE 80. AGE OF CF#1/Anl [Anl 66] MALES AT INDUCING CONCEPTION

Number	Youngest age, days	Average age, days	Range, days
66	40	48.9	40-78

TABLE 81. RESULTS OF MATING FOR CP/Anl [Anl 66], CN-1/Anl [Anl 66], AND C57BL/6Anl [Anl 66] MALES AND FEMALES

Strain	Females			Males		
	Number observed	Youngest age, days	Average age, days	Number observed	Youngest age, days	Average age, days
CP/Anl [Anl 66]	5	23	28	3	44	48
CN-1/Anl [Anl 66]	4	25	25	7	43	46
C57BL/6Anl [Anl 66]	4	34	40	3	41	42

PROGRESS REPORT

Table 80 gives the final results of studies with the CF#1/Anl [Anl 66] male. The youngest age at which a male is capable of inducing conception is 40 days. The average is 48.9 days, with a range of 40-78 days. If three males are eliminated from these data, the range becomes 40-69 days.

Table 81 gives the results of matings to date within the other three strains. The adult × adult matings in these three strains produced conceptions within 0-5 days in all cases, except for one CN-1 mating. In this instance, conception occurred 17 days after mating.

CONCLUSIONS

The final figures for the CF#1/Anl [Anl 66] ma. do not differ greatly from those reported previously.⁽¹⁾ The age at which males of the other three

strains are capable of inducing conception corresponds closely to the age for the CF $\times$ 1/Anl [Anl 66] male. The youngest age at which females of the CN-1/Anl [Anl 66] strain can conceive is 25 days, reported here, as compared to 20 days reported last year. The CP/Anl [Anl 66] female reaches sexual maturity a little earlier: 23 days reported here and 19 days reported last year. The difference in the ages reported this year and last is due to the new method used and small sampling observed this year. Females from both of these strains appear to conceive at a younger age than that reported for the CF $\times$ 1/Anl [Anl 66] female (26 days). The C57BL/6Anl [Anl 66] female

reaches sexual maturity much later (34 days) than females of other strains.

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ANIMAL VITAL STATISTICS COMPUTER PROGRAM

Thomas E. Fritz, Robert J. Flynn, Jeanne A. Pagels, and John W. Williams

PURPOSE AND METHODS

The animal vital statistics program, utilizing computer systems for data recording and processing, is directed toward developing a more efficient method of maintaining rodent breeding records, and toward developing a less laborious method of obtaining a critical analysis of the breeding efficiency of the various breeding colonies. It is also used for the collection and analysis of data on the incidence of disease in both the animals in the breeding colonies and those in experiments. These statistics are useful not only for the laboratory animal disease research program, but also for evaluating the health status of the animals used in the Division's research programs.

PROGRESS REPORT

The computerized rodent breeding record system has been used for approximately three years and has been described in recent reports.^(1,2) The data accumulated through the use of this record system are at present being used to study the influence on productivity of various factors, such as light intensity and cycle, temperature, age, and intervals between litters. These studies are described elsewhere in this report.^(3,4)

The computerized program to evaluate and tabulate data from the necropsy record keeping system has been in operation for over a year and has proved effective. An example of the form used to record data for this program is shown in Figure 182.

During the past year a computerized system for storage and retrieval of data on routine laboratory screening procedures (quality control) on breeding

and stock animals has been put into operation. This program provides a continuous and readily accessible record of the incidence of certain pathogenic or potentially pathogenic intestinal and respiratory microorganisms and external and internal parasites. The form used to record data for this program is shown in Figure 183. An example of the use of this program is found in our studies on murine pneumonia⁽⁵⁻⁷⁾ for which it was desirable to know the incidence of *Pasteurella pneumotropica* in apparently healthy stock animals.

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B1M-105 (2-66)

230

CODE		
1	2	3
0	4	5

CODE	
4	5
1	3

Accession Number				
33	34	35	36	37

TO

Accession Number				
33	34	35	36	37

QUALITY CONTROL REPORT

Date				
7	8	9	10	11

Cage Number			
15	16	17	18

Mating/Exp. No.				
19	20	21	22	23

Treatment /Grp. No.				
24	25	26	27	28

Animal No.			
29	30	31	32

Date Born				
40	41	42	43	44

Date Died				
46	47	48	49	50

Age			
52	53	54	55

Assignee		
57	58	59

Reason	
60	
☐	1 Dead
☐	2 Sick
☐	3 Moribund
☐	4 Cull-weight
☐	5 Cull-non-prod.
☐	6 Screening
☐	7 Neoplasm
☐	8
☐	9 Biopsy
☐	10

Status	
61	
☐	1 Experimental
☐	2 Stock
☐	3 Sample
☐	4 Breeding-adult
☐	5 Breeding-young
☐	6 Retired Breeder
☐	7
☐	8
☐	9
☐	10

Strain		Source	
13	14	38	39
☐	0 1	☐	0 1
☐	0 2	☐	0 2
☐	0 3	☐	0 3
☐	0 4	☐	0 4
☐	0 5	☐	0 5
☐	0 6	☐	0 6
☐	0 7	☐	0 7
☐	0 8	☐	0 8
☐	0 9	☐	0 9
☐	1 0	☐	1 0
☐	1 1	☐	1 1
☐	1 2	☐	1 2
☐	1 3	☐	1 3
☐	1 4	☐	1 4
☐	1 5	☐	1 5
☐	1 6	☐	1 6
☐	1 7	☐	1 7
☐	1 8	☐	1 8
☐	1 9	☐	1 9
☐	2 0	☐	2 0
☐	2 1	☐	2 1
☐	2 2	☐	2 2
☐	2 3	☐	2 3
☐	2 4	☐	2 4
☐	2 5	☐	2 5

HISTORY:

NECROPSY:

Sex
56

No. of Animals		
62	63	64

Speciman Examined			
65	66	67	68

No. Positive		
69	70	71

Results									
72	73	74	75		76	77	78	79	80

Fig. 183.—Quality control report

THE SUPPLY AND MAINTENANCE OF DEFINED ANIMALS FOR THE DIVISION'S RESEARCH PROGRAM: STATUS OF THE COLONY

Robert J. Flynn, Thomas E. Fritz, Calvin M. Poole,
Ronald W. Camden, and Patricia C. Brennan

PURPOSE AND METHODS

High quality research requires high quality animals maintained in such a way that the research has the best chance of successful completion. A description of the methods used to produce such animals and to maintain them in a state of health are summarized elsewhere in this report. ⁽¹⁻³⁾

PROGRESS REPORT

The number and kind of animals produced and maintained in the Division's facilities during the past fiscal year are shown in Table 82.

The studies for which these animals were used may be determined by reference to other parts of this report. Animals produced within the Division's facilities are designated with the suffix Anl.

TABLE 82. ANIMAL INVENTORY

Species		Inventory as of June 30, 1967	Total acquired July 1, 1966- June 30, 1967	Total produced at Argonne July 1, 1966-June 30, 1967
Common name	Scientific name			
Dog	<i>Canis familiaris</i>			
Beagle		712	0	162
German shorthaired pointer		1	0	0
Mongrel		1	0	0
Mouse	<i>Mus musculus</i>			
Germfree		82	160	330
SPF		12,803	0	57,706
Conventional		12,060	194	31,168
Rat	<i>Rattus norvegicus</i>	3,380	1,243	25,161
Guinea pig	<i>Cavia porcellus</i>	2	12	0
Rabbit	<i>Oryctolagus cuniculus</i>	93	330	0
Chinchilla	<i>Chinchilla laniger</i>	31	25	6
Ground squirrel	<i>Citellus tridecemlineatus</i>	43	400	0
Syrian hamster	<i>Mesocricetus auratus</i>	514	57	554
Chinese hamster	<i>Cricetulus barabensis</i>	1,042	0	1,267
Monkey	<i>Macaca mulatta</i>	1	0	0
Chicken	<i>Gallus domesticus</i>	381	1,400	0
Frog	<i>Rana pipiens</i>	0	811	0
Axolotl	<i>Siredon mexicanum</i>	22	0	0
Snail	<i>Helix aspersa</i>	66	0	100
Grasshopper	<i>Melanoplus differentialis</i>	400	200	4,000
Multimammate mouse	<i>Rattus (Mastomys) natalensis</i>	713	0	459
	<i>Mastomys erythroleucus</i>	32	0	16
Cotton rat	<i>Sigmodon hispidus</i>	86	0	100
Pack rat	<i>Neotoma albigula</i>	7	0	0
Tropical red squirrel	<i>Sciurus granatensis</i>	2	0	0
Chipmunk	<i>Tamias striatus</i>	7	5	0
Flying squirrel	<i>Glaucomys volans</i>	4	0	0
African tree rat	<i>Thamnomys surdaster</i>	1	0	3
Roundtailed muskrat	<i>Neofiber alleni</i>	2	3	0
Brush mouse	<i>Peromyscus boyleyi</i>	60	0	25
Golden mouse	<i>Peromyscus nuttalli</i>	4	0	5
Cotton mouse	<i>Peromyscus gossypinus</i>	31	0	22
Florida mouse	<i>Peromyscus floridans</i>	23	0	15
Whitefooted mouse	<i>Peromyscus leucopus</i>	362	0	467
California mouse	<i>Peromyscus californicus</i>	216	0	183
Canyon mouse	<i>Peromyscus crinitus</i>	39	0	30
Cactus mouse	<i>Peromyscus eremicus</i>	26	0	17
Hazel mouse	<i>Muscardinus avellanarius</i>	3	3	0
Tunisian jird	<i>Meriones shawi</i>	0	2	1
	<i>Meriones libycus</i>	0	2	0
Garden dormouse	<i>Eliomys quercinus</i>	0	2	2
Common dormouse	<i>Glis glis</i>	0	7	0

No research programs were curtailed for a lack of animals; no studies were prematurely terminated because of uncontrolled disease or improper care.

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Theoretical Biology

DYNAMICS OF CELLULAR POPULATIONS. COMPUTER ANALYSIS OF BONE MARROW AND BLOOD CELL KINETICS

George A. Sacher, Fumiaki Sato, and Ernesto Trucco

PURPOSE AND METHODS

The erythroid as well as the myeloid cell population of the marrow (and of the blood, for mature cells) is represented by a sequence of three compartments: proliferative, maturing, and functional. The model also includes a compartment of stem cells, assumed to be pluripotent (a stem cell may produce either erythrocytes or leukocytes). Appropriate equations are written for each compartment and solved on the CDC-3600 computer of the Applied Mathematics Division, and we gratefully acknowledge the programming carried out by Jerry J. Kaganove of that Division.

The equations have feedback terms regulating the proliferative activity and the transit of cells through the maturation compartment, according to the level of functional cells present at any time. Spatial competition between the two cell types in the marrow is also considered. For reasons of simplicity, the lymphoid elements and the thrombocytes are excluded from the present model. A special term is introduced in the equations to account for the depression of stem cells (and the resulting effects on the other compartments) under various types of continuous or fractionated irradiation.

PROGRESS REPORT

The numerical values to fit the parameters of the model are taken mostly from available data on the mouse, which provide appropriate, though incomplete, information. The data for mouse are used because, although more complete knowledge can be found in the literature for other species, such as the t, our experimental results will be obtained from BCF₁/Anl [Anl 66] mice. The present estimates still contain a great deal of uncertainty, not only in the total number of cells for each compartment, but also in the mechanisms of feedback action regulating the

functions of the whole system, as well as in the characteristics governing the kinetic properties of cellular proliferation and transit through every stage of the life-span. For these reasons, the theoretical work must proceed by trial and error. Two preliminary systems of equations were formulated and solved on the computer. In each case, the solutions showed some unsatisfactory features that were subsequently modified. At present, a third set of equations is being processed by the Applied Mathematics Division. Generally speaking, this exploratory work is aimed not so much at achieving complete agreement with experimental data, but rather at obtaining a qualitative picture of how the bone marrow behaves under normal conditions and how it responds to radiation stress.

CONCLUSION

The theoretical work outlined in this report is meant to be part of a larger project (in collaboration with R. J. M. Fry, D. Grahm, F. S. Williamson, and M. Miller), which will include a detailed analysis of bone marrow injury and recovery in mice following fractionated exposures to gamma irradiation. At this time, however, only a pilot experiment on a small number of mice is being performed. The computer output obtained to date has demonstrated that it is fruitful and feasible to simulate, and perhaps predict, certain aspects of bone marrow behavior from an adequate system of kinetic equations. This conclusion is further supported by the interesting work of Okunewick and Kretchmar⁽¹⁾ which is similar to ours in some respects.

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DYNAMICS OF CELLULAR POPULATIONS. COMPUTER SIMULATION OF PERCENT LABELED MITOSES CURVES AND COLLECTION FUNCTIONS

Ernesto Trucco, Peter J. Brockwell,* R. J. Michael Fry,
M. Donald Maclaren,* and George A. Sacher

PURPOSE AND METHODS

The main features of this project are outlined in the last annual report.⁽¹⁾ We consider a cellular population growing exponentially by binary fission, and we assume that the distributions, F_i , of the

PROGRESS REPORT

A number of simulated percent labeled mitoses curves and collection functions have been plotted. As an example, Figure 184 shows one of our results (crosses), compared with Barrett's curve (solid line)

FIG. 184.—J. C. Barrett's simulation of a percent labeled mitoses curve (solid line, taken from Figure 2 of Reference 3) and simulation with our program (crosses), obtained from assumptions matching those of Barrett's Figure 2.

random variables X_i are known. Here X_i ($i = 1, 2, 3, 4$) is the time that each cell spends in the i th phase of its life cycle (G_1 , S , G_2 , or M). From such distribution functions we calculate the age density functions for each phase, $n_i(t, a)$. These, in turn, enable us to determine $N_i(t) = \int_{a=0}^{\infty} n_i(t, a) da$, the expected number of cells in the i th phase, and $N(t) = \sum_{i=1}^4 N_i(t)$, i.e., the total number of cells in the population. Finally, we obtain the quantities $p_i = N_i(t)/N(t)$, and $g_i(a) = n_i(t, a)/N_i(t)$, that are required to initiate the computer program. Complete details for the calculations of the age density functions are given in a recent publication.⁽²⁾

At present, the theoretical analysis of percent labeled mitoses curves is being carried out by several investigators, for example J. C. Barrett⁽³⁾ and M. Takahashi.⁽⁴⁾ Their methods are somewhat similar, though not identical, to ours.

from Figure 2 of his paper.⁽³⁾ In this case we tried to duplicate Barrett's assumptions as closely as possible, and, accordingly, our computer output is quite similar to his. We believe, however, that for exponentially growing populations our procedure is an improvement over that used by Barrett, although in practice this difference has little discernible effect on the simulated percent labeled mitoses curves.

CONCLUSIONS

The preliminary phase of this study is now finished. However, a complete analysis of the results obtained so far must still be performed, and, therefore, we have not yet drawn any general conclusions from our work (see, for example, those of Takahashi⁽⁴⁾).

Further research must deal with several questions that are still open for investigation. Most important among these are possible statistical correlations between the duration of the four phases in the life

* Applied Mathematics Division.

cycle and the effect of partial synchrony on the shape of the percent labeled mitoses curves.

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IONIZING RADIATION AND BEHAVIOR

Dale D. Morris, Jeanne A. Pagels, and Merlin H. Dipert

PURPOSE AND METHODS

The general aim of this program is to study the effects of ionizing radiations on behavior in mice. The procedure is completely automated to ensure rigid experimental control. Experiments are programmed by a solid state behavioral programming system which allows four experimental chambers to be operated simultaneously. Various experimental programs currently in use are wired on program boards which can be interchanged in several seconds. Several research programs can, therefore, be run concurrently, and the utility of the system is greatly enhanced. The system is unique in that results are recorded by a data acquisition system which is on-line with a computer. Thus data are immediately sorted, manipulated, and analyzed while the experiment is in progress, allowing the results obtained to be used to direct the experiment.

PROGRESS REPORT

Currently being initiated is a study of the effects of X irradiation on reaction time in laboratory mice. Each animal is placed in a conditioning chamber, and an electric shock is programmed every 3 sec. Each lever press will delay the next shock for 20 sec. The animal learns to press the lever with a response-response interval of less than 20 sec to avoid receiving the shock. The frequency distribution of the interresponse time is measured in 2-sec class intervals. Typically, most responses occur within a few seconds of the previous response. Animals are run until a stable baseline is obtained. These animals are then irradiated and changes in the distribution of the interresponse times are recorded.

During the course of the experiment, shocks, responses, time interval signals, and error signals are recorded on magnetic tape by the data acquisition system. The stored information is coded using nine bits (binary digits). The shocks and responses are coded using the first seven bits. Time intervals em-

ploy the first eight bits with the eighth bit acting as a time flag. The time clock is variable and can be set to indicate a time base which is consistent with the experiment in progress. A 1 in the ninth bit position represents an error signal. Several hours of information are usually recorded at one time on one tape pack.

In order to analyze this information by computer methods, an interface unit was built by the Applied Mathematics Division and connected to the N register of the GE 225 computer. Through this device the data is transferred from the magnetic tape of the data acquisition system to a magnetic tape on the GE 225 computer. This process operates under program control and has been described previously.⁽¹⁾ The resulting computer tape consists of data in variable record lengths with each record containing the events which have occurred within one time base interval. A block diagram of the experimental operations is presented in Figure 185.

Three computer programs have been written which address the computer magnetic tape. These programs are mainly in FORTRAN supplemented with VFAP (Valley Forge Assembly Program), which is used to read the variable record lengths from the magnetic tape. Program I is a special tape dump which produces a listing of the coded data as recorded directly on tape. This program served mainly as a debugging tool during the development of the interface unit and control program which transfers data from the data acquisition system to the computer system. The special tape dump program is kept on hand whenever a printed copy of the raw data is needed, although it is not standard practice to produce such listings since the data are usually analyzed immediately and condensed by Program II.

The objective of Program II is to determine the number of shocks received by an animal and to form frequency distributions of interresponse times occurring in class intervals of two seconds. Each fre-

FIG. 185.—Block diagram of experimental operations

FIG. 186.—Time base diagram

quency distribution describes one hour of events. One tape record is examined at a time with (1) shocks tallied for specific boxes, and (2) responses assigned to specific class intervals based on the record number being examined, the time base assigned, and the results of events which occurred previous to the events being examined. An example follows.

Assume

Tape record i contains $T_1, T_2, R_{11}, S_{21}, S_{31}$

Tape record $i + 1$ contains $T_3, T_4, S_{41}, R_{12}, T_5,$
 R_{31}

Tape record $i + 2$ contains T_6, T_7, R_{21}, R_{41}

where T_j represents the j th time signal which defines the duration of time within one time base for all events which follow this signal until the next time signal T_{j+1} .

R_{k1} represents the k th response received from box 1.

S_{mn} represents the m th shock received by an animal in box n .

An example on a time scale is presented in Figure 186. In order to calculate the time between response R_{11} and R_{21} , the average time elapse within one time base record for R_{11} and R_{21} must be determined. These values are designated at ATE_1 and ATE_2 , respectively and computed as follows:

$$ATE_1 = TB \frac{(T_1 + T_2)}{256} - TB$$

$$ATE_2 = TB \frac{(T_6 + T_7)}{256} - TB$$

where TB is the time base.

The time between responses, TBR , is determined by

$$TBR = (TB - ATE_1) + (\Delta C) (TB) + ATE_2$$

where $\Delta C = (\text{Record number of } R_{11}) - (\text{Record number of } R_{21}) - 1$

$$\Delta C = (i + 2) - i - 1$$

$$\Delta C = 1.$$

This program yields for immediate use at the end of each daily experimental session a printout containing frequency tables of the interresponse times and percent of total responses which occurred within each class interval for each of the experimental subjects. The number of shocks each animal receives is also recorded.

The frequency distribution of the interresponse times is also punched on IBM cards. These cards are used in groups of five experimental sessions with Program III which determines means, variances, standard deviations, and standard errors for each

class interval. In this fashion it is possible to give a statistical representation of the stability of each animal's baseline behavior. After a stable baseline is established, Program III is used to determine changes in baseline behavior as a function of variables (X irradiation) introduced into the experimental situation.

CONCLUSIONS

The above system has been developed and tested and is now operational. Data collection is now under way. New programs are currently being developed which will allow for automatic control, data collection and data processing of a variety of behavioral experiments.

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REFLECTION OF GENETIC AND ENVIRONMENTAL CONTROL OF WEIGHT IN THE DOMESTIC FOWL

Sylvanus A. Tyler and S. Phyllis Stearner

PURPOSE AND METHODS

When male, Mt. Hope white leghorn chickens obtained from Kerr Chickeries, were raised under different environmental stresses (Argonne animal facilities and Colorado Agricultural Experimental Station), a difference in their mean rate of growth during the first 170 days after hatching was observed. In a strain of white leghorn, produced and grown at the Colorado Agricultural Experimental Station, males grew faster than females. The rate of accretion in Mt. Hope white leghorn embryos was distinctly higher than growth after hatching. These observations provoke several questions, among which are the following:

- (1) Can a quantitative assessment be made of the influence of genetic and environmental parameters on the rate at which birds grow?
- (2) Is the growth of the chick merely an extension of the pattern of growth followed by the embryo; or, does the evidence at hand favor a mechanism that switches the bird, at hatching, to entirely different growth-controlling kinetics?

Answers to these questions are pertinent to studies of the bird under experimentally imposed duress, and in particular, after an insult with ionizing radiation.

Descriptions of the population of birds considered in this study are as follows.

1. Male Mt. Hope white leghorn chicks, obtained from Kerr Chickeries, Frenchtown, New Jersey, were received two days after hatching. (These birds are designated in this report as "ANL" chicks.) On arrival at Argonne they were housed in electrically heated brooders until four weeks old. For the next six weeks, they were kept eight to a cage of about 520-square-inch floor space. Thereafter they were caged individually. All facilities were air conditioned; food and water were always available. Starting 40 days after hatching, the gross weight in grams of each chick was recorded at approximately 30-day intervals. In a separate experiment, 100 male ANL chicks were weighed at 2, 8, 15, 22, 29, 36, and 44 days of age. Their mean weight was determined at each of these ages.

2. Fertile eggs from white leghorn stock were obtained from Creighton Brothers, Warsaw, Indiana. They were incubated at Argonne in a standard, automatic egg incubator maintained at a temperature of 100°F and at a relative humidity of 60%. Each day, from the second through the 20th day of incubation, eggs were selected at random and opened until ten viable embryos were obtained. The embryos were

TABLE 83. MEAN AND STANDARD DEVIATION OF ESTIMATED GOMPERTZ CONSTANTS FOR INDIVIDUAL WHITE LEGHORN CHICKENS

Group	Sex	No. of animals	A_{45}		α		W_{45}, g		A_{45}/α		W_{∞}
			Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	
ANL	M	47	0.0338	0.0058	0.0220	0.0033	563	89	1.54	0.14	2626
ANL-CSU	M	93	0.0340	0.0037	0.0212	0.0055	484	74	1.65	0.23	2520
CSU	M	89	0.0282	0.0038	0.0180	0.0055	521	52	1.62	0.21	2497
CSU	F	48	0.0226	0.0032	0.0148	0.0033	461	37	1.57	0.21	2123

weighed after removal of all extra embryonic membranes and their mean daily weight was determined.

3. One hundred newly hatched, male Mt. Hope white leghorn chicks were shipped directly from the Kerr Hatchery in New Jersey to the Colorado Agricultural Experimental Station, Fort Collins, Colorado; there they were raised in floor pens until they were 170 days of age. The experiment was then terminated. Weights were recorded weekly. This group of Kerr birds is referred to as "ANL-CSU" chicks.

4. Male and female chicks, progeny of the single comb, white leghorn breeding flock at the Colorado Agricultural Experimental Station were hatched and raised at the Station. After hatching they were kept in an electrically heated brooding battery until they were four weeks old. Then they were placed in air conditioned floor pens until they were seven months old. Roughly 1000 male and female chicks were housed together; food and water were obtained from communal troughs. After seven months, these birds were caged individually. They were weighed at weekly intervals for the first 16 weeks after hatching and weighed thereafter, at 30-day intervals to 800 days of age. (The designation given to this group is "CSU" chicks.)

The Gompertz function [equation (1)] is used as the descriptive model of growth and estimation of the characterizing constants, as determined by iterative, least-square procedures; these are given in Table 83.

$$W(t) = W_{45} \text{Exp} \left\{ \frac{A_{45}}{\alpha} [1 - \text{Exp}(-\alpha t)] \right\}, \quad (1)$$

where $W(t)$ is weight at age t and W_{45} , A_{45} , and α are constants of the function.⁽¹⁾ W_{45} is the weight in grams attained at age 45 days. A_{45} is the specific growth rate at age 45 days, and α is the proportional rate of decay of the specific growth rate.

To clearly demonstrate differences between the pattern of growth in the embryo and in the young chick, the following linearizing transformation of Eq. (1) is used:

FIG. 187.—Mean weight versus age of ANL male chicks

$$\ln \left[\frac{1}{W} \left(\frac{\Delta W}{\Delta t} \right) \right] = \ln A_0 - \alpha t. \quad (2)$$

$\Delta W/\Delta t$ is a difference quotient, and A_0 represents the specific growth rate at hatching. To insure constancy in meaning of the specific growth rate (A), a suitable transformation of t is required that depends on the relationship of the hatch date to the time span of weight data investigated.⁽²⁾

PROGRESS REPORT

The average increase in weight with age for the ANL, CSU, and ANL-CSU male chicks is plotted Figures 187, 188, and 189, respectively. Since the ANL and ANL-CSU birds are drawn from the same line, the hatch weights of the two groups are presumed to

FIG. 188.—Mean weight versus age of CSU male chicks

FIG. 189.—Mean weight versus age of ANL male chicks raised in the environment of CSU chicks.

be identically distributed. At comparable ages after hatching, ANL chicks are consistently heavier than ANL-CSU chicks (Figures 187 and 189); but two of the Gompertz constants, A_{45} and α (Table 83), are essentially the same for these groups. If we tentatively assume that A_{45} and α reflect indigenous characteristics of the strain of bird, then A_{45} will express the impingement of the environment on the bird. This line of reasoning suggests that the influence of the environmental factor is reflected in the value of W_{45} . Since the Gompertz constants were determined by using only weight data at ages greater than 40 days, there is a suggestion that the environmental reaction on weight is most effective at ages less than 40 days.

The Gompertz constants for male CSU chicks are different from those for ANL chicks. The difference between the values of A_{45} and α could be attributed to differences in strain. But the Gompertz description of three additional sets of published weight data on various strains of male chickens gave an A_{45} and α for each set that were statistically compatible with these constants for ANL chicks.

The change in specific growth rate with time for both ANL chicks and embryos is shown in Figure 190. The transformation of variables, described by equation (2), facilitates this demonstration by affording a way to portray the Gompertz function as a straight line. Note that at time $t = 0$ (hatch day), an

FIG. 190.—Logarithmic transform of specific growth rate versus time from hatch day.

abrupt change in slope is evident between the trend followed by the embryo and the trend established by the young chick. Additional evidence in support of this finding has been gathered.

CONCLUSION

At this time, the weight data already surveyed merely suggest possible answers to the questions posed. It is hoped that the synthesis of additional data will indicate more clearly the plausible assump-

tions required to resolve problems toward which this investigation is directed.

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MAINTENANCE OF A BIOMEDICAL DATA BANK BY A GENERAL COMPUTER ALGORITHM

Jeanne A. Pagels and Merlin H. Dipert

PURPOSE AND METHODS

Several research programs in the Division produce data in such large quantities that it is not feasible to examine a logical tape record of these data by periodic merge-update techniques and standard recall procedures. With a large data bank, data handling efficiency demands that only the portion of the bank that requires periodic perusal is scanned and recalled. However, data of various types and of different experimental sources, whether active or inactive, must be collectively retained for future extension, recall, interrogation, and analysis. Our concern here is the construction and maintenance of such a permanent, open-end file.

We consider the following requirements for a storage scheme to be essential to efficient maintenance of a data bank:

- (1) It provides compact storage of data.
- (2) It permits a simple and direct means of updating or extending on demand the data bank.
- (3) It affords an unambiguous protocol for manipulating all classes and types of data in the bank.
- (4) It incorporates techniques for easy recall of specific data sets.
- (5) It enables any section of the data bank to be correlated with any other section.

A general algorithm that meets these requirements has been developed and is now in use in this Division. The algorithm is based on a block-storage technique, using magnetic tapes. The software used in the development of this storage scheme consists of FORTRAN and VFAP (Valley Forge Assembly Program) and the FORWARD SORT/MERGE GENERATOR.

FIG. 191.—Section of a data bank on tape

PROGRESS REPORT

In our design of a storage scheme for a collection of data, the fundamental element of the data bank is the information unit; this unit equates directly with a quantitized experimental event. The information unit, as stored on magnetic tape, is partitioned into 4 sections (see Figure 191). Section 1 registers the position, or location, at which the information

FIG. 192.—Flow of storage, rearrangement and recall operations of a data bank.

unit can be found in the data bank on tape. Section 2 describes, in coded form, the type of event that is recorded. For instance, the code 02 might mean that the information recorded in the unit describes a litter born to a particular strain x of mating number y on date z , while code 03 may designate the removal of the litter from the parents and the reason for removal. Section 3 contains general information common to all information units in the data bank. Section 4 contains the specific information descriptive of the event. This information comprises from one to several items. The information unit with the largest number of data items (based upon the information type, section 2) determines the length of all information units. Space not used by a specific information type is filled with zero values. Since the first three sections of an information unit contain the same type of data and are recorded in the same locations, these data may be sorted and rearranged in any desired fashion with the specific variables carried along during the sorting procedure.

The assemblage of information units on magnetic tape is in the form of blocks, with an inter-record gap between blocks. According to the requirements of the FORWARD SORT/MERGE GENERATOR, all blocks must contain the same number of information units. (Figure 191 illustrates a section of a data bank on magnetic tape.) The advantage of the block, as opposed to the unit record method of tape storage, is

the space saved through the reduction of $\frac{3}{4}$ -inch inter-record gaps. Storage of six records, containing twenty data items each, by the unit record technique would occupy approximately 5.4 inches of tape. By the block method of storage the same information resides in 1.6 inches of tape. Organization of a data bank in this manner establishes a versatility and flexibility in addressing the information to be rearranged and interrogated for direct recall, manipulation, and mathematical analysis.

The storage of the rodent breeding system, as reported by Fritz, Dipert, and Flynn,⁽¹⁾ does not maintain inactive records on tape. In order to analyze the entire rodent breeding colony, the block storage system consisting of 1.2 million data items is used. Four different types of information units are recorded: (1) mating description record, (2) litter record, (3) litter removal record, and (4) mating removal record. Using the FORWARD SORT/MERGE GENERATOR, this data bank has been rearranged into two different orders for retrieval of such information as (1) litter size vs. parity, (2) number of animals weaned vs. parity, (3) time intervals between parities, (4) breeding efficiency vs. temperature, and (5) breeding efficiency vs. age of the female at mating.⁽²⁾ Figure 192 describes the general operations for storage, rearrangement, and recall of a data bank.

CONCLUSION

A method has been developed to store information in large quantities at minimum cost in a data bank on magnetic tape. This information can be updated periodically and selectively rearranged for investigation and mathematical analysis.

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TIGHTENING THE CLOSED-LOOP CONTROL OF AN ANIMAL COLONY

Merlin H. Dipert and Sylvanus A. Tyler

PURPOSE AND METHODS

The increasing complexity of modern experimental design demands a comparable increase in number, precision, automatic data-recording ability, and storage capacity of the diverse instruments upon which the research scientist depends. This thrust constantly involves the scientist in a myriad of "housekeeping" chores that include the organization, management, reduction, and analysis of the experimental data produced. As the data-productive capabilities of his instrumentation increase, it becomes more imperative that a computer be listed among his equipment inventory for the purpose of assuming some of the necessary "executive" functions.

In some experiments, the computer has successfully operated as the sole control unit—receiving the data produced, and regulating or altering experimental conditions and future data production based on prior information received. This procedure is called a "closed-loop" operation. At present, experimental research in the life sciences cannot be designed for complete control by a computer, and the intervention of man at numerous places in the loop must be tolerated. Thus, attention is continually directed toward improving the efficiency of the man-machine interface as a step toward a tighter automatic control of the experiment. In other words, our aim is to reduce constantly the executive function of man (see Figure 193) by delegating it to executive computer routines. This paper summarizes the accomplishment of one attempt to increase the efficiency of our closed-loop control by computer of a large colony of beagles and rodents. In this report a specific example of the transfer of control from man to computer is given.

PROGRESS REPORT

Let us assume that an ordered, active file is maintained on all animals. Our purpose is to construct a weekly schedule of events. These events are categorized as (1) fixed age events (weaning, birth weight, vaccinations, etc.), (2) interval events (weighing, cleaning teeth, etc.), and (3) assigned events (postoperative procedure, entry into an experiment, cage assignment, etc.).

Suppose a fixed age event is to occur at age (X) of the animal. Then the age (X_0) of the animal at the beginning of the scheduled week is determined. If the age (X)

FIG. 193.—Closed-loop control of an animal colony

satisfies the condition, $X_0 \leq X \leq X_0 + 6$, the event and its date $D(X)$ are punched on a card which becomes an order to be executed. The date $D(X)$ which occurs N days after a given date can be calculated by "table-look up" using a Julian calendar. However, Dipert, et al.⁽¹⁾ devised an algorithm for using the Gregorian calendar to determine time between dates, which does not involve the use of a table in computer memory. This algorithm has been extended to establish the date $D(X)$ which is N days after some date $D(X_0)$ where N can be either positive or negative. For the fixed age event, $0 \leq N \leq 6$. For an interval event, the interval is added to the age at which the event last occurred. This case is then treated as a fixed age event. To determine whether an assigned event lies between two given dates, the dates are written in decimal notation as $YY MM DD$ and by subtraction the result is obtained. In practice a daily schedule is constructed on Thursday for the week starting the Saturday following. Control parameters that specify how often or at what age to activate certain events are read into the program at object time.

The computer run to update the colony and construct the schedule is made on Thursday. The cards are punched to initiate each of the three types of events. They are then sorted and listed by activity and associated date. This listing, which defines the order for

carrying out events such as weighing, vaccinating, etc., is given to the animal care supervisor. As the tasks are performed, only the pertinent information is recorded on the order sheet. If a measurement is taken, the amount is entered; if a task not involving measurement is performed, a check to indicate its accomplishment is entered. To record the occurrence of an event (completion of a task) for which a measurement was taken, that measurement is key punched on the original order card. Other types of information are entered by gang-punching. Thus the prepunched card and the machine-filled form become a major feature of the process. This procedure accomplishes a great saving in time for both computer and animal care personnel.

Dosimetry

BIM 130. A COMPUTER PROGRAM FOR NEUTRON-GAMMA RADIOBIOLOGY

Norman A. Frigerio and Michael H. Branson

PURPOSE AND METHODS

Many problems arise in theoretical and experimental radiobiology which are difficult to handle by simple approximations, by transport theory, or even by experimental methods. Since the physical laws governing particle behavior are well known at the atomic level, the difficulty arises in applying these laws at the macroscopic levels. Yet, it is precisely the macroscopic averages and distributions which have always been of primary interest to the radiobiologist. In recent years interest has turned, as well, to problems of microdosimetry, i.e., to interactions at the cellular and molecular levels. In this domain transport, approximation, and experimental methods are even more difficult to apply than at the macroscopic level. Thus, good computational methods are sorely needed as adjuncts to experiment, not only to provide guides for good experimental design but also to permit interpolation and extrapolation of experimental results into regions difficult to examine experimentally.

Over the last decade we have assembled computer programs intended to serve the computational needs of the radiobiologist as widely as possible. Since these needs extended from shielding and personnel safety problems, through those of radiological and radiobiological exposures, to those of microdosimetry, we turned to the Monte Carlo (MC) method.^(1, 2) This method has the advantage of permitting great problem flexibility and of affording highly detailed information, even at the microscopic level. Despite the tendency

CONCLUSION

A method is described that increases the effectiveness of automatic control of the activities associated with an animal colony by assigning to the machine and the man the function which each does best. The tracking of time, assignment of activities, and checking on completion of tasks is given to the computer, while the professional and technical tasks of animal experimentation are left with man.

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of MC to be expensive in terms of both programming and machine time in comparison with simpler, "one-problem" methods, we decided that its flexibility would outweigh this disadvantage in the long run. Certainly MC could not be recommended in cases where a one-problem approximation or transport approach would do, especially if an MC program were not available and programming had to be done *de novo*.

The decision for MC in our case, however, was justified by subsequent experience. The computations needed for the Janus facility redesign (*vide infra*), for example, could probably not have been accomplished in the time available without the flexibility provided by the MC program. In addition, with the development of subsequent generations of computers, another factor entered to reduce the running time disadvantage of MC. From our initial, simple cylinder programs for the GEORGE computer* to our more recent BIM 130A programs for the 360/75 system* the cost for runs of comparable complexity and precision has decreased by 10^3 , while the available information per unit cost has increased by 10^5 . This has resulted largely from an unexpected tendency of modern computer design to favor the type of internal operation characteristic of MC.

For the last year a program, coded BIM 130, has been operational on the CDC 3600 computer.* With the completion of tests against independent computa-

* Applied Mathematics Division.

tions and, most important, against experiment, this version may be considered ready for general use. Since considerable effort was invested in rendering it simple to use, it may be considered available for immediate use even by users not otherwise skilled in computer techniques. Copies of the program and an instruction booklet are available on request. It provides a flexible computational tool adapted to the complex geometric, chemical composition, and particle source spectrum problems which so often face the radiobiologist—one which computes not only dose and LET distributions, but also solutions for problems of particle spectra, reflection, exposure, shielding, activation, dosimeter response, and other parameters. Thus, it provides the physical information needed for the design and utilization of experimental facilities and for the interpretation of experimental results.

BIM 130, described below, utilizes the CDC 3600* to capacity.

A more recent version, BIM 131, is operational on the IBM 360/75* system at about one-eighth the unit computational cost of BIM 130. In addition, the limits on geometrical regions, nuclides, histogram and spectral energy bins have been raised by a factor of four. When optimization of BIM 131 is complete, it is expected that improvement by another factor of 4 will have been obtained.

PROGRESS REPORT

Program Description

The approach used is that of the phenomenological (or "complete analog") Monte Carlo computation in a real, finite, Cartesian space. The space of interest is designated region No.1, and defined by its (x, y, z) size. Any particles leaving this region are considered "out," and their space-energy vectors are recorded as they leave.

Within this space up to 14 other regions may be specified, each by its (x, y, z) position and size, by its shape, and by its chemical (or nuclide) composition. Each of these regions is assumed to be in thermal equilibrium with region 1. Regions may have any shape from rectangular parallelepipeds through circular or elliptical cylinders to spheroids/ellipsoids. Regions may be of any size, including zero, in one or two dimensions, so that planes, discs, points, lines, etc. may be defined. Regions may also lie within one another in any order and, in this way, one may build up objects of complex shape and size.

Regions above No. 15 are at present reserved for sources. Their sizes, shapes, and temperatures are specified independently, but their compositions are

those of the region(s) in which they lie, or through which they pass. Particle emission from a source region may be specified as isotropic, parallel to any axis, or with any set of arbitrary $f(\theta)$ distributions, so long as the latter are supplied in tabular or equation form. Similarly, energy may be specified as monoenergetic, Maxwell-Boltzman distribution of given temperature, Watts-Rule fission spectrum for ^{235}U , or any arbitrary spectrum which is furnished tabularly. Throughout the program tabular data are interpolated as needed.

The geometry and composition established, the user enters the number of histories to be followed, their type (neutron or gamma), the temperatures, and the maximum time to be allowed. At the same time, he chooses any of several dozen options (*vide infra*) and enters the needed nuclear data as to differential scatter [elastic, inelastic, and nonelastic as $F(E, \theta)$] and capture, for γ and n .

The program then establishes a source particle vector from this information, computes the point of interaction (or exit) by continuous allowance for the composition of the regions intersected by the vector's extension. From weighted probability tables, based on the input information, the particular element (nuclide) of interaction is chosen, as well as reaction type, and thermal motion vector of the atom (nucleus) heading toward the point of collision. If photoelectric capture occurs, the photon history is complete. The program records the space-energy description of the event and returns for a new source particle. If neutron capture occurs, the space-energy description of the event is computed, the recoil energy of the nucleus is computed, and the neutron history goes on to trace all secondary, tertiary, etc. particles (e.g., capture gamma rays) until these are captured or exit. Only then does the program return for a new neutron.

If scatter occurs, the local description of the event will contain the energy change of the recoiling nucleus. Since the incoming particle may be at a lower average energy than the nuclei of the irradiated object, such change can be + or -. A thermal neutron at 20°C, for example, would gain energy on elastic collision with the atoms of living tissue at 37°C. The program computes this change in energy signs, and the changes in angular momenta.

From the necessary differential cross sections, center-of-mass $\rightleftharpoons$ laboratory transformations, functions for chemical binding (very important for H), etc., the program determines the leaving vector of the scattered particle. Any secondary particles (e.g., gamma rays from inelastic scatter) are now followed along their leaving vectors until they either exit or are captured. The program returns to the scattered

* Applied Mathematics Division.

particle, computes the position of the next interaction, etc. This continues until the specified number of histories have been run, each history being the complete record of the interactions of one source particle and all of its daughters. During this process, the details of each interaction may be stored on external tape for later review at the option of the user.

Output is now processed according to the options selected by the user at the time of input. One of these options concerns the sections which are to be printed, so that any or all of the sections below may be omitted as desired.

The first output section prints all of the input data, e.g., cross sections, geometry, source, options, etc. There are so many possible combinations of input parameters that failure to include all input data, along with output, would quickly lead to piles of output that are impossible to identify. Then, too, a print of input data allows the user to check for unintentional errors or modifications in his input instructions.

The system then prints a summary page for quick reference, which gives the count and energy totals for scatters, captures, gammas, first collisions, source particles, and noninteracting passages for each region. The numbers and energies of interactions are then broken down according to element (nuclide) for each of the regions and for each of the interaction types. Energy spectra for each region are then given, for neutrons and for gammas, broken into 128 energy bins each. Spectra are under control of several options and may be inbound, outbound, interaction, interacting, first collision, subsequent collisions, etc. The energy bins are specified by input cards and may have any values, uniform or arbitrary. Exit spectra are then given for the six boundary planes of region No. 1 ($\pm x, \pm y, \pm z$).

LET number/energy spectra for 24 independently chosen regions are then printed out for each of the possible interaction types, for each element (nuclide), and into 50 LET bins. As with energy spectra, bins are selected by input cards and may have any arbitrary distribution.

At this point, the energy histograms for neutron and for gamma interactions are printed out as a (x, y, z) matrix whose elements may number 6144 or less. On command, any number of arbitrary sets of elements within this matrix will be summed and printed.

Following this, any, or all spectral elements may be operated upon by sets of input values, and the results are printed out for each of the 6 exit planes and each of the 15 regions. "First collision factors" could be entered, for example, for comparisons with values computed by the program, or the response

curves of particular detectors could be entered so that both real and apparent predictions can be made.

Finally, the times for various sections of the computation are printed out for guidance in future runs. In the event that the user, on inspection, decides that a different set of options would have been preferable, he may simply rerun the interaction tape through the output section of the program. In this way, a rerun is avoided at a great saving in time.

Overall run time depends on the complexity and, especially, the size and composition of the system. At one extreme, the problems associated with the energy deposition in air of a gamma source high above a level field will be processed at a rate of $>10^4$ photons per minute. At the other extreme, the complete moderation and capture of 14-MeV neutrons in a very large block of Pb might require one minute per neutron. Viewed from the standpoint of interactions, however, speed is constant at about 10^4 interactions per minute. The two cases above differ primarily in the number of interactions that each source particle undergoes between emission and capture/exit.

The program is set up as a complete analog, and no "Russian roulette" or other forms of importance selection are employed. Options are provided, however, which have the effect of increasing relative efficiencies for particular problems.

Various combinations of options can be used to achieve good precision for cases of interest without excessive use of machine time. To minimize errors of input information by the user, a diagnostic subroutine is included which tests input information for mathematical and physical reasonableness.

Gamma Tests.—One of the options permits the program to use gammas as the source particles. Except for their particular physical differences, the program handles both γ and n the same way. This area of overlap covers about 85% of the total program. Because of the relatively complete theoretical and experimental data available for gamma rays, the gamma option seemed a good starting point for program testing. All the usual "first collision" equations were tested against the program, and agreement was well within statistical expectations (e.g., $\pm 0.1\%$ for 10^6 histories).

The program was then set up to compute the dose to a spherical shell of standard air at 273.16° K , 200 cm thick, located 10^5 cm from a small ^{137}Cs source. The value obtained for $\mu e/\rho$, $0.0294 \text{ cm}^2 \text{ gm}^{-1}$, is well within experimental error of the accepted value, $0.0293 \text{ cm}^2 \text{ g}^{-1}$.

The experimental situation of Sinclair⁽³⁾ was then set up for computation, i.e., a 20-cm glass-enclosed sphere of FeSO_4 dosimeter solution at 293° K , located

100 cm from the ^{60}Co source of our High Level Gamma Room. Although ^{60}Co gamma rays have a mean energy of 1.25 MeV, the output spectrum computed by the program yielded a mean energy of 1.04 MeV because of scatter and absorption within the source; 1.04 MeV is in good agreement with literature values for such sources.⁽⁴⁾ The computed value for average rads per roentgen was 0.758 vs. an experimental value⁽³⁾ of 0.75, once again well within experimental error. Time for analysis of problem and punching of input data was 4 man-hours. Run time on machine was 150 minutes.

Parenthetically, agreement with the incomplete analog Monte Carlo computations of Ellet^(5, 6) was well within expected error. The discrepancies, seen in Table 84, are those to be expected for Ellet's use of 3.41×10^{23} electrons cm^{-3} in an incomplete analog Compton routine, instead of the true value of 3.31×10^{23} for the composition used. With this correction, they lie within Ellet's statistical precision of about $\pm 2\%$.

Gamma Usage.—A problem was presented to us by D. Grahn, of this Division, *in re* the ^{60}Co irradiator used by the Atomic Energy Commission for overseas demonstrations. A load of cattle feed had been irradiated for a cooperating scientist, but average doses and dose rates were not measured; only dose rates at points on the periphery of the irradiation cylinder were measured. The geometry was complex in the extreme. The irradiator consisted of a cylindrical steel tank, water filled, set on a concrete slab and surrounded by packed sand. Within the water phase were a lead storage pot, five ^{60}Co slab sources arranged asymmetrically about the irradiation cylinder, and a quantity of lights, pipes, controls, etc. The cylinder itself was only partly filled with feed and weighted down with a bag of lead shot to make it sink.

Computing directly from the given Curie strengths of the five sources, we obtained an average absorbed dose rate of 17,600 rads/hr. At the same time, this computation gave peripheral dose rates within $\pm 3\%$ of those measured, an interesting check on the ability of the program to go from source strength directly to dose distribution, despite complex geometry, extensive scattering, etc. About 12-man-hours and 380 minutes of machine time were required for this test.

Neutron Tests.—Beyond simple, first-collision, tests (all to better than $\pm 0.5\%$) good experimental data in the neutron field have been difficult to obtain. In consequence, we set up an experimental "acid test" fission counter response as a function of position in a phantom human head, irradiated with polyenergetic neutrons from anisotropic, finite sources of size com-

TABLE 84. ABSORBED FRACTION^(a) TO 70-kg PHANTOMS OF ROSSI TISSUE-EQUIVALENT MATERIAL, $E_\gamma = 0.662$ MeV

Shape	Source	A.F., ^(a) Ellet ^(5, 6)	A.F., ^(a) BIM 130
Sphere	Point	0.651	0.635
Sphere	Uniform, distributed	0.469	0.453
Ellipsoid	Point	0.475	0.466
Ellipsoid	Uniform, distributed	0.348	0.336
Ellipsoid cylinder	Uniform, distributed	0.361	0.354
Ellipsoid cylinder	Point	0.481	0.470

^(a) Absorbed Fraction = Absorbed γ energy/emitted γ energy.

FIG. 194.—Response of ^{235}U fission counter in a human head phantom. Simple lines were obtained experimentally; circles are points computed. Experimental curves were obtained using a 3-keV Li target and the $^7\text{Li}(p, n)$ reaction with the phantom centered at 0° and subtending a total arc of $\pm 70^\circ$. The solid curve (182 keV) was obtained at a proton energy of 1.963 MeV for an energy spread of 182 keV at 0° to 61 keV at 70° . The dashed curve (1555 keV) was obtained at a proton energy of 3.245 MeV. Neutron energies ranged from 1555 keV at 0° to 1115 keV at 70° for the $^7\text{Li}(p, n)^7\text{Be}$ group and 1080 keV at 0° to 747 keV at 70° for the ^7mBe group.

parable to both detector size and relaxation length. Since fission counter response is, itself, nonuniform with neutron energy, an additional complication was added. Results are shown in Figure 194. This test is

TABLE 85. COMPARISON OF COMPUTED DEPTH DOSES IN A 30 X 60-CM CYLINDRICAL PHANTOM FOR 2.5-MeV NEUTRONS

Penetration, cm	Neutron dose, rads $n^{-1} \text{ cm}^2 \times 10^{-9}$		Gamma dose, rads n^{-1} $\text{cm}^2 \times 10^{-9}$	
	BIM 130	Snyder ⁽²⁾	BIM 130	Snyder ⁽²⁾
1.5	3.7	3.1	1.76	1.7
4.5	3.15	2.8	2.40	3.2
7.5	2.30	1.4	2.84	3.5
10.5	1.60	1.0	3.41	3.7
13.5	1.12	—	3.16	—
15.0	—	0.7	—	2.6
16.5	0.72	—	2.51	—
19.5	0.47	0.3	2.17	2.6
22.5	0.32	0.3	1.62	1.5
25.5	0.21	0.2	1.21	0.8
28.5	0.14	0.05	0.70	0.4

FIG. 195.—Midline flux density distribution 343 cm (= 0 cm) from GLEEP reactor thermal column, with and without a 20 X 30 X 60-cm man-phantom. Experimental results shown as lines, computer results as circles.

very sensitive to experimental values, especially at low or high values of x . Thus, the discrepancies, although small, probably reflect inadequate control of scatter during the experiment. Although a more precise rerun is planned, Figure 194 shows very acceptable agreement with experiment for a priori computation done under very complex conditions. About 40 man-hours, and 480 machine-minutes, were required.

Comparison was also made with the single cylinder of Snyder⁽²⁾ for an incident neutron energy of 2.5 MeV. Results are shown in Table 85. Differences are only those to be expected from the incomplete analog nature of Snyder's program. Thus, BIM 130 doses are consistently a little higher because of more complete histories for neutrons moderated into lower energy regions.

Since chemical binding effects are so important for low energy neutron interactions with tissue a com-

parison was made with the recent experiments of Boot and Dennis.⁽⁷⁾ They exposed an elliptical cylinder of tissue equivalent liquid to slow neutrons from the thermal column of a reactor, measuring thermal fluxes within and without the cylinder with a small BF₃ counter. Experimental results (lines) are compared with our computed ones (circles) in Figure 195. Results of other computer calculations, of the incomplete analog type, are reported by Boot and Dennis.⁽⁷⁾ Lack of agreement for these, however, ran to an order of magnitude or more instead of the few % shown in Figure 195. For Figure 195 BIM 131 was used, with an investment of 4 man-hours and 70 machine-minutes.

Neutron Usage.—To date, chief usage has been in conjunction with Janus dosimetry and redesign.⁽⁸⁾ In this connection, it has been nearly invaluable, permitting computations and design where experimental determinations would not have been feasible. Where comparisons of measurement and computation have been possible in Janus, BIM 131 has acquitted itself well. Average neutron energies at one meter were computed as 0.27 and 1.2 MeV under two conditions, and measured as 0.25 and 1.2 MeV, respectively. Further tests are planned in Janus, as well as extensive cross checks against the detailed, and complex, experiments of Lawson and Watt.⁽⁹⁾

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GEOMETRIC FACTORS FOR GAMMA-RAY DOSIMETRY IN CYLINDERS

Lincoln B. Hubbard and Frank S. Williamson

PURPOSE AND METHODS

In dosimetric analyses of biologic problems, the use of simplified geometries and distributions can be of value since each dosimetric calculation is no longer unique. For some biologic geometries the sphere is an adequate approximation, but for others it is not sufficient.⁽¹⁾ Right cylinders are more likely to approximate to the shape of animals. These cylinders, although having a high degree of symmetry, cannot be integrated exactly to find the geometric factors, except for points lying on the curved surface or the axis.^(1,2)

In a uniform body the absorbed dose rate to a point is

$$\frac{\Delta D(r)}{\Delta t} = f \Gamma a \int \frac{e^{-\mu R}}{R^2} dV_k.$$

The derivation of this and the definition of the quantities are quoted elsewhere in this report.⁽³⁾ The integral in this case has been defined as the "geometric factor"⁽⁴⁾

$$g(r) = \int \frac{e^{-\mu R}}{R^2} dV_k.$$

The average absorbed dose is related to the average geometric factor by

$$\bar{g} = \frac{1}{V} \int g(r) dV$$

as

$$\frac{\Delta \bar{D}}{\Delta t} = f \Gamma a \bar{g}.$$

In general, the dosimetric analysis of uniform cylinders requires some form of numerical integration. For those interested in the results, but not wishing to carry out the analysis, the numerical data available to date are inadequate. The table by Loewinger, Holt, and Hine⁽⁵⁾ purporting to present values of average geometric factor for cylinders is mislabeled and, in fact, has been shown to apply to values of g for an axial point at the end of the cylinder.⁽⁶⁾ Focht, Quimby, and Gershowitz⁽⁷⁾ have presented a table of average geometric factors for use with $\mu = 0.028$ in cylinders, but comparison with the method to be described has revealed errors in this table for radii of less than 10 cm.

There is a difficulty in presenting this type of data. The geometric factor is a function of the five variables: R (radius of cylinder), L (length of cylinder), r (radial position of the point), z (axial position of the point), and μ (absorption coefficient). The average geometric

factor is a function of the three variables L , R , and μ . Thus, in the direct presentation, the average geometric factor must be plotted as a family of curves or in a series of tables. The five-parameter display of the geometric factor for individual points would be so voluminous that it is unlikely to be attempted.

The three-variable average geometric factor was presented in the literature^(5,7) for a single value of μ and a two-dimensional table for L and R . Such a table, if correct, would be usable only for that single value of μ and would, in general, require a double interpolation over L and R .

It is desirable to devise a method of reducing the number of parameters involved in this problem in order to present the data efficiently.

PROGRESS REPORT

The geometric factor and average geometric factors have both been reduced to functions of two variables fewer by extracting the "size" and the absorption. First, consider the size effects in the particular case of the linear absorption coefficient being zero ($\mu = 0$)

$$g_0(r, z, R, L) = \int_0^{L/2} \int_0^R \int_0^{2\pi} \frac{r_k d\phi dr_k dz_k}{(z - z_k)^2 + r^2 + r_k^2 - 2rr_k \cos \phi} \quad (1)$$

We can change to dimensionless forms by the following definitions:

$$\alpha = r/R \quad \beta = z/L \quad \gamma = L/R$$

$$S_0(\alpha, \beta, \gamma) = \frac{1}{\sqrt[3]{\gamma}} \int_0^{1/2} \int_0^1 \int_0^{2\pi} \frac{\alpha_k d\phi d\alpha_k d\beta_k}{\gamma(\beta - \beta_k)^2 + \frac{1}{\gamma}(\alpha^2 + \alpha_k^2 - 2\alpha\alpha_k \cos \phi)} \quad (2)$$

The four-variable function $g_0(r, z, R, L)$ with the dimensions of length can be equated to the normalized geometric number S_0 , a dimensionless function of three variables:

$$g_0(r, z, R, L) = \sqrt[3]{R^2 L} S_0\left(\frac{r}{R}, \frac{z}{L}, \frac{L}{R}\right). \quad (3)$$

Similarly, the average geometric factor $\bar{g}_0(L, R)$ with no absorption is

$$\bar{g}_0(R, L) = \sqrt[3]{R^2 L} \bar{S}_0\left(\frac{L}{R}\right). \quad (4)$$

The preceding definition of S_0 separates the variable "size" from the geometric factor.

Although the size variable can be separated out exactly, the separation of the absorption variable cannot be made exact. An approximate method is possible. Formally, an exact absorption function can be split off from the geometric factor,

$$g_\mu(r, z, L, R) = g_0(r, z, L, R) f_\mu(r, z, L, R), \quad (5)$$

but this formal separation yields no real advantage because the absorption function $f_\mu(r, z, L, R)$ depends on all the position variables. The absorption function has the form $f_\mu = 1 - A_\mu$, where A_μ is the fraction absorbed by including μ . For volumes where the Marinelli, Quimby, Hine definition of the geometric factor is valid, $A_\mu \ll 1$. Thus, f_μ represents a first-order correction to the geometric factors, and errors in A_μ represent second-order terms.

The approximate absorption function that is most easily handled is the average spherical absorption

$$F^s(\mu R) = \frac{\bar{g}_\mu^s(R)}{\bar{g}_0^s(R)}. \quad (6)$$

This is⁽³⁾

$$F^s(x) = \frac{4}{3} - \frac{1 + 2x}{2x^4} \cdot \left(e^{-2x} - 1 + 2x - 2x^2 + \frac{4}{3}x^3 \right). \quad (7)$$

It can be expected that the absorption in a cylinder would not be significantly different from that in a sphere of the same volume; consequently "the effective radius," R^s , for use in these formulae is given by

$$R^s = \sqrt[3]{\frac{3}{4} R^2 L}.$$

FIG. 196.—The average normalized geometric number for right circular cylinders

FIG. 197.—Comparison of three different calculations for the average normalized geometric number for right circular cylinders with $L = R$ and $\mu = 0.028 \text{ cm}^{-1}$.

While this absorption function is approximate, it must be close to the exact function, which cannot be expressed in closed form.

CONCLUSION

We have a method which reduces by two the variables in the calculation of geometric factors and hence simplifies their presentation. In final form the geometric factors for right cylinders are:

$$g_{\mu}(r, z, R, L) \cong \sqrt[3]{R^2 L} S_0(r/R, z/L, L/R) F^s \left(\mu \sqrt[3]{\frac{3}{4} R^2 L} \right), \quad (8)$$

$$\bar{g}_{\mu}(R, L) \cong \sqrt[3]{R^2 L} \bar{S}_0(L/R) F^s \left(\mu \sqrt[3]{\frac{3}{4} R^2 L} \right). \quad (9)$$

The function $F^s(x)$ is given in formula (7) and the values of S_0 and $\bar{S}_0$ can be calculated with the programs discussed elsewhere.⁽³⁾ The value for $\bar{S}_0$ is given in Figure 196.

In Figure 197, we present the result of a calculation of the average normalized geometric number $\bar{S}_{\mu}$ for right circular cylinders with $L = R$ and $\mu = 0.028 \text{ cm}^{-1}$. A check of the accuracy of this approximation was made using the "cube model" computation⁽³⁾ directly for cylinders of $L = R = 10 \text{ cm}$ and 20 cm . The points show excellent agreement. As a further check, data obtained from the table of Focht, Quimby, and Gersho-

witz have been used to calculate values of $\bar{S}_0$, which are included in Figure 197. Agreement is good for values of $L = R > 10 \text{ cm}$, but these data are, unexpectedly, smaller at $L = R < 10 \text{ cm}$. We have no information concerning the computational details of their table, but it seems improbable that, as $L = R \rightarrow 0$, the value of $\bar{S}$ should tend to zero, when the value of $\bar{g}/R$ for a sphere tends to a value of 3π .

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GAMMA-RAY DOSIMETRY OF NONUNIFORMLY DISTRIBUTED INTERNAL EMITTERS

Lincoln B. Hubbard and Frank S. Williamson

PURPOSE AND METHODS

Determination of the spatial distribution of gamma radiation dose in a biological subject which is burdened with a radionuclide presents a complex problem. In general, it is desirable to have the capability of calculating, for any point in the tissue, the gamma dose which is produced by the disseminated radionuclide.

In the general case, the calculation must take into consideration the following factors:

- (1) The biological subject is not a regular geometric shape.
- (2) The tissue is inhomogeneous in both density and atomic composition.
- (3) The radionuclide is nonuniformly distributed in the tissue.

In principle the Monte Carlo procedure, in which nuclear disintegrations and the histories of the resultant photon interactions with the tissues are randomly

sampled, would appear to offer an ideal method. Unfortunately, the number of parameters required in the general problem, when coupled with the necessity of accumulating statistically-significant numbers of photon histories, leads to unreasonably long computation times and may even overtax the memory capacity of the present generation of computers.

The procedure we have chosen is nonstatistical in nature. The biological specimen is divided into a large number of elementary volumes (cubes, for example), and the laws of gamma-ray attenuation and scatter are invoked to calculate the contribution at each element from other elements. Such a calculation is well suited to the capabilities of a digital computer.

As is well known, an elementary volume ΔV_k , of activity $a(\mathbf{r}) \Delta V_k$, may be regarded as a point source situated at its center $\mathbf{r}_k$. If there is no scattering medium present, it produces an exposure rate to the target volume element centered at $\mathbf{r}$ of:

FIG. 198.—The ray from the cube (3, 10) to the cube (6, 8). It is assumed that the radioactive source and the resulting doses are point distributions at the center of their respective cubes. The absorbing properties of a cube are assumed uniform over the cube volume.

$$\frac{\Delta X(\mathbf{r})}{\Delta t} = \Gamma \frac{a(\mathbf{r}_k)}{R^2} \Delta V_k \text{ roentgens.} \quad (1)$$

Γ is the specific gamma-ray constant for the isotope, and R is the effective separation of points $\mathbf{r}$ and $\mathbf{r}_k$. The absorbed dose rate at this point $\mathbf{r}$ is:

$$\frac{\Delta D(\mathbf{r})}{\Delta t} = f(\mathbf{r}) \frac{\Delta X(\mathbf{r})}{\Delta t} \text{ rads,} \quad (2)$$

where the conversion factor is:

$$f(r) = 0.869 \left(\frac{\mu_{\text{en}}}{\rho} \right)_{\text{medium}} / \left(\frac{\mu_{\text{en}}}{\rho} \right)_{\text{air}}.$$

μ_{en} is the mass energy absorption coefficient and ρ is the density.

If a uniform intermediate medium is present which only scatters photons out of the beam from $\mathbf{r}_k$ to $\mathbf{r}$, then the exposure and absorbed dose rate are modified by an attenuation factor, $e^{-\mu R}$, where μ is the linear attenuation coefficient, also known as the total absorption coefficient for the gamma rays. Thus

$$\frac{\Delta D(\mathbf{r})}{\Delta t} = f(\mathbf{r}) \Gamma a(\mathbf{r}_k) \frac{e^{-\mu R}}{R^2} \Delta V_k. \quad (3)$$

In general, a medium will also scatter photons into the target volume at $\mathbf{r}$. This multiple scattering into the target volume is very complex. Allowance has sometimes been made for this contribution by the use of a "build-up factor" or an "effective μ ."

For sizes considerably less than the mean free path of the photons ($1/\mu$), the effects of multiple scattering are not important, and they will not be considered here.

When multiple scattering is neglected, the absorbed

dose rate at a target $\mathbf{r}$, from a point source $\mathbf{r}_k$, in a medium with a variable attenuation is

$$\frac{\Delta D(\mathbf{r})}{\Delta t} = f(\mathbf{r}) \Gamma \frac{a(\mathbf{r}_k) \Delta V_k}{R^2} \exp \left[- \int_{\mathbf{r}_k}^{\mathbf{r}} \mu(s) ds \right]. \quad (4)$$

For a distributed source in this inhomogeneous medium, the absorbed dose rate is

$$\frac{\Delta D(\mathbf{r})}{\Delta t} = f(\mathbf{r}) \Gamma \int_{V_k} \frac{a(\mathbf{r}_k)}{R^2} \cdot \exp \left(- \int_{\mathbf{r}_k}^{\mathbf{r}} \mu(s) ds \right) dV_k. \quad (5)$$

PROGRESS REPORT

Programs have been written to implement the calculation of various quantities involved in these dosimetric calculations. These programs are written mainly for the IBM 360-50/75, although some are written for the CDC 3600. Since we are interested in nonuniform body calculations, the geometry that we use is the Cartesian space, i.e., we divide a body into elementary cubes. For arbitrary bodies, the form of calculation is

$$\frac{\Delta D(r)}{\Delta t} = f(r) \Gamma \sum_k a(r_k) \prod_i (1 - \mu_i X_i) \frac{1}{R^2} \Delta V_k. \quad (6)$$

X_i is the ray length traversing the i th cube. R is considered as the effective distance between cubes; this is approximated as $R^2 \cong (\mathbf{r} - \mathbf{r}_k)^2 - 0.25 c^2$, where c is minimum distance between cube centers. To carry out this calculation correctly, it is necessary to calculate the "self dose" of a cube. This is achieved by subdividing a cube of a fixed volume, with the requirement that the result be independent of the number of subdivisions. Similar corrections were achieved in like manner for the 26 next-neighbor cubes.

Figure 198 shows the path of a sample ray from one cube to another in a two dimensional plane. The contribution to the dose at (6,8) from (3,10) is

$$\begin{aligned} \frac{\Delta D}{\Delta t} (6,8/3,10) &= f_{6,8} \Gamma a(3,10) (1 - \mu_{4,10} X_{4,10}) \\ &\cdot (1 - \mu_{4,9} X_{4,9}) (1 - \mu_{5,9} X_{5,9}) (1 - \mu_{5,8} X_{5,8}) \\ &\cdot \frac{1}{12.75 c^2}. \end{aligned} \quad (7)$$

(Note that absorption in the source and target volumes are neglected.) Here the distances in each cube are

$$\begin{aligned} X_{4,10} &= X_{5,8} = 0.300462 c, \\ X_{4,9} &= X_{5,9} = 0.901388 c. \end{aligned}$$

Using given values of $f(r)$, Γ , ρ_i , and μ_i the program calculates the terms in this manner and adds them up.

Using this technique, we are able to calculate dose

distributions and the average doses to bodies of arbitrary shape, arbitrary isotope burden, and arbitrary composition. There are only two major limitations. Unlike Monte Carlo calculations, there are no statistics and so the time limitation depends only upon the number of cubes into which the geometries are divided. For reasonable computer times, of the order of ten minutes, the number of cubes in a fully arbitrary calculation is about 1,000; for uniform and symmetric bodies this can be speeded considerably to about 20,000 cubes in the same time. Time demands increase approximately as $N^{7/3}$ for arbitrary calculations or $N^{5/3}$ for uniform bodies, where N is the number of elementary volumes chosen. The other limitation is the assumption that all scattering effects are adequately handled by assuming exponential absorption. This approximation is good for small animals, such as mice, but is considerably weaker for moderate sized animals, such as dogs, and is not valid for larger animals, including man. This is due to the large build-up of scattered radiation. A method of correcting for this scattering contribution, which is compatible with the present technique, has been developed.⁽¹⁾

CONCLUSION

A number of tests of the validity of the cube model have been undertaken. The dose distributions and average dose for a sphere can be calculated for uniform spheres. For uniform bodies, the absorbed dose calculation simplifies:

$$\frac{\Delta D(r)}{\Delta t} = f\Gamma a \int \frac{e^{-\mu R}}{R^2} dV_k. \quad (8)$$

For a sphere of radius R ,⁽²⁾

$$\begin{aligned} \frac{\Delta D(r)}{\Delta t} = f\Gamma a \left\{ \frac{4\pi}{\mu} + \frac{\pi(R^2 - r^2)}{r} \right. \\ \cdot [Ei(-\mu R - \mu r) - Ei(-\mu R + \mu r)] \\ \left. + \frac{\pi}{r\mu^2} [(1 + \mu R - \mu r)e^{-\mu(R+r)} \right. \\ \left. - (1 + \mu R + \mu r)e^{-(R+r)}] \right\}, \end{aligned} \quad (9)$$

where $Ei(x)$ is the exponential integral

$$Ei(-x) = - \int_x^\infty \frac{e^{-t}}{t} dt.$$

The average absorbed dose rate for spheres is

$$\begin{aligned} \frac{\Delta D}{\Delta t} = f\Gamma a R \left\{ \frac{4}{3} - \left(\frac{1}{2} + \frac{\mu R}{\mu^4 R^4} \right) \right. \\ \left. \cdot \left(e^{-2\mu R} - 1 + 2\mu R - 2\mu^2 R^2 + \frac{4}{3} \mu^3 R^3 \right) \right\}. \end{aligned} \quad (10)$$

FIG. 199.—Comparison of the cube model results with the exact values for the average value of the dose rates for spheres with $\mu = 0$. The fluctuations in volume of the model give rise to similar fluctuations of less amplitude in the average dose rates.

FIG. 200.—Comparison of the cube model results for the absorbed dose rates at points inside a sphere with the exact formula. The value of μR chosen is 2.5.

The validity of representing a volume as a series of cubes has been extensively tested. This can be done with considerable confidence by comparing the cube model results against the exact integration of the sphere.

In Figure 199, the results of the model for a sphere with $\mu = 0$ are compared with the exact value for the average absorbed dose rate ($3\pi f\Gamma aR$). It can be seen that the cube model gives excellent results (within 2% for spheres with the diameter at least six times the minimum distance between cube centers).

The results for individual points, rather than the average, are shown in Figure 200. The model points shown are those along a radius from the center. These, of course, are only a small fraction of the points that the program calculates, but they are properly representative. It can be seen that the approximate results are not perfect but are adequate.

Our method was intercompared with the Monte Carlo technique of Frigerio and Branson (BIM-130)⁽³⁾ for 0.66-MeV photons emitted with uniform activity within a cylinder of water 10 cm long and 5 cm in radius. The first-collision dose calculation of BIM-130 was compared with the dose derived from the cube model geometric factor;* this is appropriate since the geometric factor gives the first collision dose if the "good geometry" linear absorption coefficient is used.

* The geometric factor is defined as⁽⁴⁾

$$g = \int \frac{e^{-\mu|r-r'|}}{(r-r')^2} dV'$$

In a two-and-one-half-hour calculation, BIM-130 followed 500,000 photon histories to give an average dose of 1.89×10^{-6} rads. An eighteen-second calculation using our method gave a value for the average geometric factor of $\bar{g} = 29.75$. With $\Gamma = 3.0$ rads/mCi-hr, this is equivalent to an average dose of 1.70×10^{-6} rads.

The Monte Carlo calculation employed the atomic cross sections, whereas the cube model results were interpreted with the macroscopic variables commonly used in gamma-ray dosimetry. It is felt that this difference of basis could contribute to the discrepancy between the values of absorbed dose.

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TOXICITY AND METABOLISM OF RADIONUCLIDES

Studies of Radiation-Induced Changes

A COMPARISON OF HEMOPOIETIC RESPONSE AT THREE AGE LEVELS IN BEAGLES GIVEN NEAR-LETHAL TO LETHAL DOSES OF CESIUM-137

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PURPOSE AND METHODS

Single doses of ^{137}Cs were given intravenously to beagles when 5 months, 13 months, or 48 months of age. The average dose during the first 30 days after injection was approximately 825 rads for the younger age groups.⁽¹⁾ The excretion rate is age-dependent,⁽²⁾ being highest in the youngest dogs, so the dose rate is higher in the older dogs during the critical periods of hemopoietic recovery.

Comparable doses of ^{137}Cs were given to each of three groups of dogs when they were 5 months and 13 months of age; only one group of dogs was available at 48 months of age (Table 86). The survival ratios at the three age levels were: 10/13 when injected at 5 months, 6/13 at 13 months, and 0/5 at 48 months. All observations are based upon blood obtained by jugular venipuncture. The hematology in this phase of the ^{137}Cs toxicity program provides no

unique information per se about radiation toxicity; however, the blood study is useful to illustrate further the age-dependent factor.

PROGRESS REPORT

There are two distinct responses shown by the cellular populations of venous blood after a single large dose (3.27–4.32 mCi/kg) of ^{137}Cs was given intravenously. The initial response occurs in the first 100 days, and is characterized by extreme depression of leukopoiesis and a moderate to severe depression of erythropoiesis. There is a transitory and moderate tendency toward increase in mean erythrocyte volume between 50 and 100 days. Evidence of hematopoietic recovery is apparent after 35 to 50 days, but there were large individual variations; the recovery rate was frequently unsteady, and maxima of recovery varied significantly.

TABLE 86. BEAGLES GIVEN SINGLE LARGE DOSES OF ^{137}Cs FOR COMPARISON OF HEMATOLOGIC EFFECTS AT DIFFERENT AGE LEVELS

5-Month				13-Month				48-Month			
Group	Dog No.	mCi/kg ^{137}Cs	Death, days	Group	Dog No.	mCi/kg ^{137}Cs	Death, days	Group	Dog No.	mCi/kg ^{137}Cs	Death, days
5-I	474	3.27		13-I	390	3.34		48-II			
	472	3.31			391	3.35					
	473	3.32			436	3.42					
	471	3.34			435	3.43					
5-II	486	3.60		13-II	239	3.64			48	3.67	20
	485	3.63			243	3.65	35		71	3.73	22
	491	3.65			367	3.74	36		72	3.73	22
	487	3.70	39		369	3.77	28		69	3.74	24
	492	3.76			437	3.78			46	3.77	21
5-III	398	4.00	27	13-III	419	4.15	27				
	401	4.12	27		416	4.25	24				
	397	4.18			418	4.30	26				
	404	4.32			417	4.33	27				

After 100 days, cell production is less subject to fluctuation and may, for some classes of leukocytes, show very slow and moderate increases in absolute numbers over a period of years. In contrast, eosinophils characteristically show a significant, temporary increase between approximately 110 and 250 days. Average postinjection values for leukocytes have not recovered to preinjection levels in any of the five groups having survivors. There is less fluctuation in the average erythrocyte counts than in leukocyte counts during the total recovery period. In three groups (5-II, 5-III, 13-II) there was a reduction of about 60% in erythrocytes during the first 100 days but over-recovery is common after 300 days. The extreme of over-recovery is recorded for two individuals having a number of counts from 9 to 11 million cells per cubic millimeter. After 100 days, the mean erythrocyte volume tends to be normal or 5 to 10 cubic microns below the initial levels.

Variations due apparently to age differences are seen primarily in the survival data, but these variations are also apparent in the more rapid recovery of hematopoiesis in younger animals. In a further evaluation of the data, a comparison of the average major populations of the leukocytes can be made between the preinjection values for the 13-month groups

and the 5-month groups when they reached 13 months of age. After the 5-month groups had survived 8 months with a ^{137}Cs burden, they had approximately the same numbers of mature neutrophils (at 13 months) as unirradiated dogs at that age. The numbers of immature neutrophils are obviously lower for the dogs irradiated between 5 and 13 months. There are fewer normal lymphocytes after 8 months of irradiation by ^{137}Cs , but this difference does not appear to be significant.

CONCLUSION

In both surviving age groups, population sizes for the various classes of cells are not static, but, in general, leukopoiesis appears to be somewhat depressed, while erythropoiesis is stimulated in response to ^{137}Cs doses in the near-lethal range.

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URINARY CALCULI AND THEIR RETENTION OF RADIONUCLIDES IN THE BEAGLE DOG

Lillian V. Kaspar and William P. Norris

Twelve beagles from this colony were diagnosed with urolithiasis (urinary calculi) from 1960 through 1967; this is an incidence of about 1.5% in a total of about 900 dogs. Of these, 7 were normal, unirradiated animals: dogs 540 and 715 were from stock, 1024 was a control, 462, 467 and 530 were from the breeding colony, and 694 had been reared on a semisynthetic diet since birth. Dog 900 had been exposed to whole-body irradiation from a ^{60}Co γ -ray source. Four dogs were injected with radionuclides: 128 received ^{137}Cs , 17 and 580 were given ^{144}Ce , and 558 received a tracer dose of ^{42}K . Dog 128 has developed bladder stones on four separate occasions; each time the stones were removed surgically.

When analyzed, the stone taken from dog 694 was found to be cystine; all the remaining stones contained magnesium, phosphate, and ammonium ions. Moisture, ash, and phosphorus determinations showed these stones to be almost pure struvite or magnesium ammonium phosphate hexahydrate ($\text{MgNH}_4\text{PO}_4 \cdot$

$6\text{H}_2\text{O}$) (Table 87). This analysis was confirmed by X-ray diffraction studies* of a few selected stones.

All stones were assayed for radioactivity, using an RIDL 400-channel γ -ray spectrometer analyzer. Calculi from 4 dogs showed activity. The first set of calculi from dog 128 were found to contain 9.65 μCi ^{137}Cs , or almost the entire residual body burden of this isotope. This animal had received, in a 16-month period, three separate injections of ^{137}Cs , totaling 36.1 mCi. Thus, the retention of ^{137}Cs in the uroliths was 0.027% of the total dose. The mechanism whereby ^{137}Cs becomes incorporated into struvite calculi is considered an ionic replacement of NH_4^+ by $^{137}\text{Cs}^+$ within the crystalline lattice structure of struvite. The latter is analogous to the $\text{Cs}^+ - \text{NH}_4^+$ substitution

* Dr. Aaron S. Posner, New York Hospital, Cornell University Medical College, New York, ran and examined X-ray diffraction patterns of calculi from dogs 128, 462 and 558 and found them to be identical to struvite.

used in the separation of ^{137}Cs from other fission product wastes.⁽¹⁾

Figure 201 shows a cut section of a radioactive urolith from dog 128 and the autoradiograph made from it. Three distinct areas of labeling are present that correspond to the three injections of cesium. The dark, innermost core represents the initial injection. The second and third injections are shown by the two dark bands separated from, and surrounding, the core. The uroliths subsequently removed from this dog showed no radioactivity.

The concretions from dogs 540, 17, and 580 showed radioactivity at the tracer level, i.e., approximately $1 \times 10^{-5} \mu\text{Ci}$. The activity in the stones from dog 540 was shown to be ^{137}Cs and its accumulation was attributed to the concentration of the isotope from fallout over a period of several years.⁽²⁾ The activity in the stone from dog 17 was identified

as a mixture of ^{144}Ce , ^{154}Eu , and ^{155}Eu . The Europium isotopes were contained as radiochemical impurities in the original preparation of ^{144}Ce injected 70 months earlier. The radioactivity in the uroliths from dog 580 was due to only ^{144}Ce , since radiochemical impurities were not present in the original preparation. The incorporation of ^{144}Ce , ^{154}Eu , and ^{155}Eu in urinary calculi appears to be due to the trapping of these isotopes within the calculi as they grew, rather than to ionic substitution.

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TABLE 87. MOISTURE, ORGANIC, ASH, AND PHOSPHORUS CONTENT OF URINARY CALCULI IN BEAGLE DOGS

Specimen	Original specimen	Specimen dried at 105°C			Radionuclide present
	H ₂ O, %	P _i , %	Loss at 550°C (Organic), %	Ash, %	
MgNH ₄ PO ₄ ·6H ₂ O ^(a)	44.0	22.5	10.6	45.4	—
17	41.4	22.2	9.3	49.3	^{144}Ce , ^{154}Eu , ^{155}Eu
128A	46.2	23.2	10.5	43.1	^{137}Cs
128B	44.3	21.0	10.7	45.0	—
128C	46.4	22.0	8.6	45.0	—
128D	45.6	23.0	10.4	44.0	—
462	45.6	23.1	10.5	43.9	—
467	42.6	22.4	10.7	46.7	—
530	45.1	22.2	8.9	46.0	—
540	43.7	24.0	10.7	45.6	^{137}Cs
558	44.2	23.3	8.2	47.6	—
580	42.4	21.6	12.5	45.1	^{144}Ce
694 ^(b)	3.7	0	96.3	0	—
715	46.5	22.7	7.3	46.2	—
900	42.9	22.6	11.4	45.7	—
1024	42.7	22.3	9.7	47.6	—

^(a) Theoretical moisture, ash, and organic composition of MgNH₄PO₄·6H₂O:

^(b) Dog 694 had the cystine bladder stone.

FIG. 201.—A, Cut section of a urinary calculus removed from dog 128; B, radioautograph of the calculus showing three areas of labeling corresponding to three injections of ^{137}Cs .

THE RESPONSE OF THE BEAGLE DOG TO ^{60}Co GAMMA RADIATION. I. DETERMINATION OF THE $\text{LD}_{50/30}$ AND DESCRIPTION OF ASSOCIATED CHANGES

William P. Norris, Thomas E. Fritz, Carl E. Rehfeld, and Calvin M. Poole

The median lethal dose ($\text{LD}_{50/30}$) of ionizing radiation delivered uniformly to the whole body of dogs in a single, short exposure was $258.5 \pm \text{S.E.} = 3.5$ rads. Radiation was delivered, bilaterally, from a ^{60}Co source at ~ 15 R/min. The animals were purebred beagles—34 males and 27 females, 13 months old when irradiated.

Radiation dose was computed for each animal and expressed as the average absorbed dose in rads. The latter follows from published dosimetric data in cylindrical phantoms filled with ferrous sulfate solution.⁽¹⁾ Compensation for variations in animal thickness as it influences the average absorbed dose made a significant improvement in the relationship of dose to response.

A study of the clinical, biochemical, hematologic, bacteriologic and histopathologic response of these animals to irradiation led to the conclusion that bacteremia, secondary to granulocytopenia, was the primary cause of death in each case. Thus, the intervention of bacteremia prevents a full expression of the biologic response to radiation. The effect of such intervention on the interpretation of data related to the $\text{LD}_{50/30}$ is considered in some detail.

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THE RESPONSE OF THE BEAGLE DOG TO ^{60}Co GAMMA RADIATION. II. THE RESPONSE OF BEAGLE DOGS TO CONTINUOUS EXPOSURE TO ^{60}Co GAMMA RAYS

Calvin M. Poole, William P. Norris, Carl E. Rehfeld, and Thomas E. Fritz

PURPOSE AND METHODS

The methods and design for this experiment were presented in last year's annual report.⁽¹⁾ Briefly, the experiment involved the near-continuous exposure of beagles to ^{60}Co gamma radiation. Thirty-six dogs were exposed for a period of 100 days, or until death intervened, at exposure rates equivalent to 72, 50, 35, 24, 5, or 17 R/day. As radiation-induced deaths occurred, the decedents were replaced by dogs scheduled to be sacrificed when they had accumulated predetermined radiation exposures at rates of either 72, 50, 35, or 24.5 R/day. Clinical observations, hematologic examinations, urinalyses and necropsies of decedents were performed.

The purpose of this study was to examine the response of the beagle to protracted whole-body irradiation. Such data allow comparisons with results obtained in dogs given near-lethal doses of ^{137}Cs —an isotope which produces the approximate dosimetric equivalent of whole-body exposure. It also has the potential value of increasing the understanding of recovery mechanisms and the determination of safe radiation levels.

PROGRESS REPORT

The portion of this experiment in which dogs were kept in the γ -ray field until death, or for the entire 100-day exposure period, is summarized in Table 88. Data relevant to the decedents, both radiation-induced and sacrificed, were reported previously,⁽¹⁾ except for data pertaining to 2 deaths which occurred during the period covered by this report. These deaths (0908 and 0943) are especially notable because of the extended survival time of these dogs, 405 and 502 days, respectively. The longest survival time of the other decedents was 169 days.

Dog 0908 presented a clinical syndrome somewhat different from that of dogs observed earlier. One week prior to death, it developed a grossly swollen left upper eyelid and a hemorrhagic conjunctivitis. This condition became progressively worse with the development of an ulcer of the eyelid, frank hemorrhage from the involved tissues around the eye, and oozing blood from the conjunctiva. Subsequently, the right eye became similarly involved, but much less severely.

This dog had gradually developed a rather severe anemia after irradiation, from which it only partially recovered. During the irradiation period it developed a severe leucopenia, from which it also

failed to recover to more than 31% of preirradiation values. Three days prior to death, its red blood cell (RBC) count fell precipitously; this was attributed to hemorrhage. The white blood cell (WBC) count at this time also revealed a marked leucopenia. Although not proved, it was felt that unusual involvement of the eyes and surrounding tissues may have been precipitated by the mild trauma associated with the self-operated swinging door operated by the dog in each kennel. The eye involvement, in turn, contributed to the development of a septicemia, which resulted in death.

Necropsy findings in 0908 were primarily hemorrhages. There were moderate to marked hemorrhages of the lymph nodes. Petechiae were found in the adrenal cortex, the kidneys, the urinary bladder mucosa, the stomach mucosa, and the small intestine mucosa. There were 4 ecchymoses in the mucosa of the colon. Ecchymotic hemorrhages were also observed in the left parietal pleura. The left lung also showed consolidation of the entire apical and cardiac lobes, and half of the diaphragmatic lobe. The terminal diagnosis included pneumonia, septicemia, and hemorrhagic necrotizing conjunctivitis.

The syndrome presented by dog 0943 was a gradual, progressive anemia during the two months prior to its death. The terminal erythrocyte count was 32% of preexposure values. Septicemia developed and produced a somewhat earlier death than would have occurred in its absence. This septicemia was undoubtedly initiated by a number of intravenous injections of a tranquilizer which was followed by a purulent necrotic condition that developed in the leg in which the injections were made. The tranquilizer was administered in support of retinal photography. Increasingly severe retinal hemorrhages were observed for approximately 10 days prior to death. The necropsy findings were essentially those of septicemia and anemia. It is of interest to note that the terminal septicemia elicited a marked increase in leucocyte numbers.

The surviving 8 animals currently appear to be grossly normal except for a reduced testicular size in the males.

The hematologic response of the survivors has been followed by periodic sampling of peripheral venous blood. The WBC counts exhibited an almost immediate response and steady decline during the first 30 days of exposure. At that time, ineffective attempts at recovery were exhibited, followed by a less rapid

TABLE 88. RESPONSE OF DOGS TO PROTRACTED Co⁶⁰ γ -RAY EXPOSURE

Exposure dose, R/day	Dog No.	Date irradiation begun	Date of death	Days to death	Exposure, R	Avg. absorbed dose, rads	Exposure dose, R/day	Dog No.	Date irradiation begun	Date of death	Days to death	Exposure, R	Avg. absorbed dose, rads
72	0887	9-17-65	10-14-65	27	1944	1485	24.5	0891	9-19-65	12-20-65	92	2254	1686
	0895	↓	10-12-65	25	1800	1357		0892	↓	12-07-65	79	1936	1442
	0910	↓	10-16-65	29	2088	1608		0900	↓	3-07-66	169	2450	1864
	0932	↓	10-10-65	23	1656	1257		0942	↓	—	—	2450	1850
								0945	↓	—	—	2450	1801
Average				26	1872	1427		0948	↓	—	—	2450	1820
								0950	↓	—	—	2450	1766
50	0888	9-17-65	10-20-65	33	1650	1261		0999	↓	—	—	2450	1815
	0902	↓	10-19-65	32	1600	1210	Average				113	2361	1755
	0904	↓	10-17-65	30	1500	1163							
	0911	↓	10-26-65	39	1950	1472	17	905	9-20-65	3-05-65	166	1700	1273
	0916	↓	10-24-65	37	1850	1391		908	↓	10-30-66	405	1700	1282
	0946	↓	11-13-65	57	2850	2092		909	↓	—	—	1700	1306
	0989	↓	10-17-65	30	1500	1133		943	↓	2-04-67	502	1700	1267
	0991	↓	11-03-65	47	2350	1781		949	↓	1-27-66	129	1700	1301
Average				38	1906	1438		954	↓	—	—	1700	1251
								955	↓	2-07-66	140	1700	1253
35	0889	9-18-65	11-03-65	46	1610	1230		995	↓	—	—	1700	1268
	0893	↓	11-02-65	45	1575	1205	Average				268	1700	1275
	0907	↓	10-29-65	41	1435	1102							
	0912	↓	11-01-65	44	1540	1180	Control	944					
	0913	↓	12-01-65	74	2590	1919		964					
	0969	↓	10-29-65	41	1435	1082		974					
	0992	↓	11-25-65	68	2380	1811							
	0994	↓	11-24-65	67	2345	1782							
Average				53	1864	1414							

rate of reduction in WBC numbers for the remainder of the exposure period. The mean leucocyte count in the survivors at the end of the exposure period was approximately 20% of their preexposure values. Recovery of white cell numbers started immediately after irradiation stopped. The rate of recovery was less than the rate of decline, and the total white count failed to reach preirradiation values. The mean total leucocyte counts currently (638 days after exposure ended) are 70% of preexposure values.

The mean RBC counts in the two groups of survivors exhibited declines to approximately 80% of preirradiation values; however, the time of onset of decline and recovery differed between the 24 R and 17 R

per day exposure groups. The period of decline for the 24 R survivors occurred between 50 and 135 days after the exposure began. The 17 R survivors exhibited the decline between 100 and 180 days after exposure began. Survivors in both radiation groups showed recovery to preirradiation values at about 350 days.

REFERENCE

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CERIUM-144—TOXIC MANIFESTATIONS IN BEAGLE DOGS

Thomas E. Fritz, William P. Norris, Carl E. Rehfeld, and Calvin M. Poole

PURPOSE AND METHODS

Forty-nine purebred beagles, approximately 13 months of age, were given ^{144}Ce citrate intravenously in doses ranging from 22.8 to 253.0 $\mu\text{Ci/kg}$ body weight. Earlier reports^(1, 2) described the acute lethality of higher doses of ^{144}Ce and also emphasized the high incidence of bone tumors in dogs which survived the lower doses of this nuclide for 1000 days or longer.

PROGRESS REPORT

Since our report last year,⁽²⁾ another 10 dogs have died, or were killed because they became moribund (0580, 0585, 0583, 0564, 0519, 0518, 0021, 0017, 0031, 0026). There are now 11 survivors. Details of the doses of ^{144}Ce given to these dogs are presented in Table 89, where they are arranged in the order of the doses received (in $\mu\text{Ci/kg}$ body weight). The primary cause of death or the significant lesions as determined at necropsy or by microscopic examination are also presented.

Six of the dogs which either died or were killed during the past year had malignancies diagnosed as the primary cause of death or as the reason they were destroyed (0580, 0564, 0021, 0017, 0031, 0026). Three animals died after a protracted and progressive anemia, and the remaining animal had a massive hepatic hematoma associated with extensive hepatic degeneration and secondary renal infarction. Four of the neoplasms were osteogenic sarcomas: three were in the vertebrae; the fourth was in the scapula. The tumor in the vertebra of dog 0026 is shown in Figure 202. The other two neoplasms were a myelogenous leukemia and a hemangiosarcoma. Dog 0031, with an osteogenic sarcoma, also had a hemangiosarcoma. Two of the osteosarcomas and both of the hemangiosarcomas had metastasized at the time of necropsy. The lungs of dog 0564, containing metastatic tumors of a hemangiosarcoma, are shown in Figure 203. One of the dogs that died of anemia (0519) developed bilateral glaucoma and detached retinas, associated with intraocular hemorrhage two weeks before death.

The hemangiosarcoma in dog 0564 was particularly significant because the primary tumor was at the site of cerium injection. During injection into the saphenous vein, it was noted that some of the isotope may have leaked into the perivascular subcutaneous tissue. In subsequent weeks an obvious radiation burn with epilation, hyperpigmentation, and scarring developed at this point. At necropsy, the tissue adjacent to this area contained the only tumor that was not obviously metastatic.

All 10 animals showed some degree of liver damage both at necropsy and on microscopic examination. These changes were characterized by focal to diffuse fatty degeneration, focal necrosis, scarring and contraction of the capsule, and focal nodularity of the surface, resulting from regeneration and hyperplasia of the parenchyma. The livers of dogs 0519 and 0026 are shown in Figures 204 and 205. A review of the necropsy findings of all dogs in this experiment which survived longer than 1000 days after being injected with cerium-144 demonstrates that liver lesions are a consistent response in dogs surviving the acute lethal phase of ^{144}Ce toxicity.

CONCLUSIONS

The conclusions in our previous reports^(1, 2) concerning the cause of death and induction of disease in cerium-injected dogs hold true for the study reported here. Animals receiving doses of ^{144}Ce of more than 70 $\mu\text{Ci/kg}$ generally died within 100 days with signs of hematopoietic failure; those receiving doses of less than 70 $\mu\text{Ci/kg}$ survived more than 1000 days, after which neoplasms, predominately osteogenic sarcomas, occurred. The development of osteogenic sarcomas in three more dogs in the lowest dose group (less than 30 $\mu\text{Ci/kg}$) demonstrates that the endpoint for induction of these tumors was not reached in this experiment.

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TABLE 89. SURVIVAL OF BEAGLES FOLLOWING A SINGLE INTRAVENOUS DOSE OF Ce^{144}

Dog No.	Sex	Date injected	Age of injection, days	Amount		Days to death	Survival at 10-1-67, days	Cause of death
				μCi	$\mu Ci/kg$			
132	M	8-10-60	680	2100	253.0	17		Bone marrow aplasia
119	M	8-10-60	680	2000	248.8	18		Bone marrow aplasia
112	M	8-10-60	772	1800	153.8	23		Bone marrow aplasia
129	M	8-10-60	649	1400	150.5	33		Bone marrow aplasia
261	F	4-18-62	500	956.0	122.7	0		Euthanized
1	M	12-6-60	420	1086	120.0	26		Bone marrow aplasia
2	M	12-6-60	420	995.0	102.0	31		Bone marrow aplasia
3	M	12-6-60	420	833.0	96.4	53		Bone marrow aplasia
580	F	3-24-64	416	797.0	80.3	1198		Myelogenous leukemia
548	M	1-15-64	410	823.4	79.2		1355	
544	F	1-15-64	410	760.1	78.0	57		Bone marrow aplasia
547	M	1-15-64	410	624.6	77.9		1355	
183	M	2-11-64	75	698.0	73.4	48		Hypoplasia of bone marrow
594	F	3-24-64	406	681.0	74.0		1286	
585	M	3-24-64	416	979.0	72.8	1120		Bone marrow aplasia
595	F	3-24-64	406	819.0	72.7	46		Bone marrow aplasia
589	M	3-24-64	416	761.0	72.5	89		Bone marrow aplasia
597	F	3-24-64	406	739.0	71.0	920		Osteogenic sarcoma
598	M	3-24-64	406	953.0	69.0	56		Bone marrow aplasia
586	M	3-24-64	416	1098.0	67.1	60		Bone marrow aplasia
583	F	3-24-64	416	775.0	66.0	1191		Hepatic necrosis
565	M	2-11-64	415	705.0	64.0		1328	
578	M	2-11-64	405	629.0	63.3		1328	
516	M	11-27-63	412	606.0	62.9		1404	
520	M	11-27-63	412	589.1	62.2		1404	
521	F	11-27-63	412	571.6	62.1	560		Undetermined
564	F	2-11-64	415	619.0	60.9	1280		Hemangiosarcoma
567	F	2-11-64	408	739.0	59.9	99		Bone marrow aplasia
562	F	2-11-64	415	550.0	59.5		1328	
519	M	11-27-63	412	626.9	58.9	1133		Bone marrow aplasia
518	F	11-27-63	412	460.8	58.6	1242		Bone marrow aplasia
517	M	11-27-63	412	481.9	55.2		1404	
9	M	1-10-61	395	499.0	55.1	1158		Osteogenic sarcoma
7	M	1-10-61	395	463.0	53.0	1182		Reticulum cell sarcoma, of vertebrae
11	M	1-10-61	395	439.0	49.7	1261		Osteogenic sarcoma
12	M	1-10-61	395	410.0	49.4	955		Osteogenic sarcoma
13	M	1-10-61	395	394.0	48.3	1138		Osteogenic sarcoma
30	M	2-21-61	388	465.0	47.2	90		Bone marrow aplasia
22	M	2-21-61	410	436.0	47.1	77		Bone marrow aplasia
21	M	1-24-61	392	281.0	28.4	2441		Osteogenic sarcoma
14	M	1-24-61	396	240.0	28.1	1780		
16	M	1-24-61	396	246.0	27.8	376		Hepatic necrosis and bone marrow aplasia
17	M	1-24-61	396	281.0	27.8	2124		Osteogenic sarcoma
15	M	1-24-61	396	216.0	27.5		2441	Osteogenic sarcoma
20	M	1-24-61	392	287.0	27.4	1444		Osteogenic sarcoma
32	M	2-21-61	388	243.0	25.1	1529		Osteogenic sarcoma
31	M	2-21-61	388	198.0	24.8	2078		Osteogenic sarcoma
26	M	2-21-61	408	194.0	23.8	2102		Hemangiosarcoma
33	M	2-21-61	388	176.0	22.8		2423	Osteogenic sarcoma

FIG. 202.—The 6th cervical vertebra of dog 0026. An osteogenic sarcoma partially occludes the canal

FIG. 203.—Lungs of dog 0564. Numerous metastatic tumors of a hemangiosarcoma of the skin are shown as dark, raised nodules

FIG. 204.—Liver of dog 0519. There is marked nodularity resulting from degeneration and hyperplasia of the parenchyma. There is also thickening of the capsule and a fibrin cast on the surface (upper right).

FIG. 205.—Liver of dog 0026. There is focal degeneration of the parenchyma, thickening and contraction of the capsule, and formation of serous cysts.

RESPONSE OF THE BEAGLE THYROID TO LOCALIZED X IRRADIATION

*James A. Taylor, William P. Norris, Calvin M. Poole,
Thomas E. Fritz, and Norbert D. Kretz*

PURPOSE AND METHODS

The purpose of this study was to determine whether the thyroid glands of dogs in this colony would show damage after localized exposure to 2000 or 250 kVp X rays and, if so, to characterize these changes as a function of time after irradiation.

While the thyroid gland has been reported to be

quite resistant to ionizing radiation, one report⁽¹⁾ describes clinically recognizable thyroid disease as a delayed response to exposure of the upper body of beagle dogs to about 2000 R of 1000 kVp X rays.

Thirty beagle dogs, 12 males and 18 females, were selected from the ANL colony. They were divided into 5 experimental groups of 6 dogs per group and treated as follows:

Group	Treatment
I	Control
II	Left thyroid lobe removed surgically
III	Left thyroid lobe irradiated with 2000 R
IV	Both thyroid lobes removed surgically
V	Both thyroid lobes irradiated with 2000 R

Thyroid lobes which were to be irradiated were dissected free of connective tissue without disrupting the blood supply and were elevated sufficiently that the collimated X-ray beam did not impinge on other tissues in the neck. In unilateral treatments, the left lobe was always manipulated. The left thyroidal lobe of each of the controls was surgically elevated, sham-irradiated, and replaced. Radiation was delivered, using a 250 kVp General Electric Maximar machine, at a rate of 142 R/min.

The dogs were killed at intervals of approximately 1, 3, 6, 9, and 12 months after irradiation. Each sacrifice consisted of 5 dogs—one from each experimental group. Five are still alive. Thyroid function was evaluated by (1) repeated studies of the uptake of ^{131}I (1 μCi per test) and the subsequent rate of loss of ^{131}I from the gland, (2) by histologic examination of each thyroid lobe obtained during the experiment or when the animal was killed, (3) weight of thyroid lobes, (4) biochemical determinations of serum cholesterol and protein-bound iodide (PBI), (5) radioautographic visualization of ^{131}I deposition in the thy-

TABLE 90. PEAK UPTAKE OF ^{131}I IN THE THYROID GLANDS OF DOGS ON RESTRICTED IODIDE INTAKE

Time on diet, days	50	100	170	260	350	440
Time after treatment, days		25	95	185	275	365
Peak uptake, % of injected dose						
Treatment						
Control	21.0	46.4	52.6	60.2	65.0	66.0
Hemithyroidectomized	21.9	23.5	39.0	43.2	55.8	30.9
Unilateral irradiation	25.9	38.4	37.4	50.1	65.6	51.4
Total thyroidectomy	26.8	1.7	3.4	8.1	7.3	3.2
Bilateral irradiation	22.1	29.4	26.3	14.2	19.3	24.0

TABLE 91. RETENTION OF ^{131}I IN THYROID GLANDS OF DOGS ON RESTRICTED IODIDE INTAKES

Time on diet, days	50	100	170	260	350	440
Time after treatment, days		25	95	185	275	365
Estimated biologic half-time, days						
Treatment						
Control	8.2	7.2	5.8	4.0	25.7	32.5
Hemithyroidectomized	10.3	3.7	2.8	2.8	7.2	21.7
Unilateral irradiation	8.9	5.6	3.8	2.8	14.6	23.4
Total thyroidectomy	8.5	1.0	1.4	1.9	2.9	2.5
Bilateral irradiation	10.1	5.6	2.8	2.4	3.1	5.2

roids. Each dog was given 50 μCi ^{131}I 24 hr before it was killed.

Since previous work⁽²⁾ showed that ^{131}I uptake in the thyroid is heavily dependent on the iodide content of the diet, all animals in this experiment were fed a casein-based semisynthetic diet which contained 25 μg iodide/100 g diet. This provided a daily iodide intake of 50–75 μg for each dog. Dogs were 11 months old when placed on this diet. This occurred 75 days prior to initiation of the experiment.

PROGRESS REPORT

Data relevant to maximum ^{131}I uptake in thyroidal tissue, retention of ^{131}I in thyroidal tissue, and weights of thyroidal tissue, in dogs tested at various intervals after treatment are summarized in Tables 90, 91, and 92, respectively.

Two unexpected results have appeared. The first relates to the fact that of 27 dogs in this study whose thyroids have been histologically examined thus far, 6 have shown lymphocytic thyroiditis. This condition has rarely been recognized in dogs. The incidence of spontaneous thyroiditis in dogs in this colony has been studied in more detail, and is included in another section of this report.⁽³⁾

The second unexpected result was the dramatic increase in the weight and the altered histologic appearance of the unirradiated thyroid glands in response to a restricted dietary intake of iodide. This response also is reported in more detail elsewhere in this report.⁽⁴⁾

The latter response was fortunate, since it provided an additional demonstration of the inability of the irradiated lobes to respond to restricted iodide intake (Table 92). This difference was evident as early as 25 days after irradiation and became progressively more apparent with increasing time.

As expected from previous work,⁽²⁾ restricted iodide intake produced a major increase in ^{131}I uptake by the thyroid gland. The present work shows

TABLE 92. WEIGHTS OF DOG THYROID TISSUE WITH TIME ON RESTRICTED IODIDE INTAKE

Time on diet, days	50	100	170	260	350	440
Time after treatment, days		25	95	185	275	365
Avg. weight of one thyroid lobe, g						
Treatment						
Control	0.44	0.95	1.33	1.53	0.81	1.48
Hemithyroidectomized		0.85	1.35	1.89	1.88	1.87
Unilateral irradiation						
Unirradiated lobe		0.85	1.30	0.89	1.92	2.47
Irradiated lobe		0.49	0.27	0.25	0.26	0.40
Bilateral irradiation		0.45	0.34	0.32	0.33	0.31

that it required approximately 6 months to attain a reasonably constant value (Table 90).

Exposure of thyroid tissue to 2000 R caused a definite reduction in ^{131}I uptake (Table 90), but, in comparison to surgical removal, did not stop this uptake completely. The remaining lobe, after unilateral irradiation or surgery, was nearly able to compensate for absent or damaged tissue.

The loss of ^{131}I after its uptake in thyroid tissue, as expressed by estimations of the associated biologic half-times (Table 91), became progressively faster during the first 260 days of restricted iodide intake. After 350 and 440 days on the diet, however, the biologic half-time measured in the controls became markedly longer. This behavior was also apparent in the unilaterally treated dogs, but not to the same degree it was in the controls. The bilaterally irradiated animals could not make this transition.

The biologic half-times obtained were related to the histologic appearance of the thyroid tissue.⁽⁴⁾ When the half-times were short, the glands appeared markedly hyperplastic and devoid of colloid. However, the longer half-times seen after 350 and 440 days on the diet were associated with a reversion of the thyroid to a more normal histologic structure. This consisted of a reduction in the degree of hyperplasia, with an increase in follicular colloid. This occurred both in the controls and in the untreated lobes of unilaterally irradiated or surgically treated animals.

In no case was there clinical evidence of thyroid

insufficiency—even though other measures of thyroid function showed that 2000 R produced marked damage to the gland.

Some ectopic thyroid tissue was detected by scanning the neck region of animals for concentrations of ^{131}I . This was usually found in those dogs which had been bilaterally thyroidectomized by surgical methods.

The histologic appearance of irradiated lobes differed sharply from that of dogs on a conventional diet and also from the appearance in lobes of unirradiated dogs on limited iodide intake. Within 95 days the irradiated lobes showed atrophy of the follicular epithelium, reduction of colloid, reduction in follicular size, and interstitial and perivascular fibrosis, which progressed during the period up to 185 days after exposure. Some evidence of an attempt to repair this damage was seen by 275 days after irradiation.

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Characterization of the ANL Beagle

MEASUREMENT OF PHYSIOLOGIC PARAMETERS OF THE DOG USING RADIOTELEMETRY. FACTORS INFLUENCING BODY TEMPERATURE

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PURPOSE AND METHODS

Sublethally irradiated animals may live for years in apparent good health. The meaningful evaluation of such animals on an individual basis presents a scientific challenge which, so far, has not been successfully met. It is clear that all systems of the body may have sustained damage. We judge, therefore, that the best approach is to measure the overall capacity of the intact individual to respond to a con-

trollable stimulus. This series of reports on the toxicity and metabolism of radionuclides in the beagle dog further identifies the need for such a measurement.

Maintenance of body temperature of mammalian homeotherms within physiologic bounds requires the combined capabilities of many functional and biochemical systems. These include (1) the cardiovascular system, (2) the respiratory system, (3) the erythropoietic system, (4) certain of the endocrine functions, (5) the central nervous system, and many others. It is inviting, therefore, to propose that an

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animal's ability to maintain body temperature under somewhat stressful conditions, such as reduced ambient temperatures or reduced partial pressures of oxygen, may be a valuable, nondestructive measure of its overall physiologic competence. Such a technique should provide much valuable data relevant to changes that occur during aging or after sublethal doses of ionizing radiation.

This evaluation requires an essentially continuous record of body temperature of an animal maintained under controlled conditions. In dogs, this record is best obtained with surgically implanted, miniaturized, radio-telemetry devices—this is especially so since many physiologic parameters, including temperature, in the dog are quite sensitive to excitement. In this colony, dogs typically demonstrate abnormal respiration, increased pulse rate, elevated blood pressure, and body temperatures up to 40.0°C when they are examined by routine procedures.

Suitable telemeters, capable of stable performance over reasonably long periods of time, are not available. Therefore, they were developed in collaboration with members of the Electronics Division of this Laboratory. They were designed to allow their adaptation to transmission of data from physiologic parameters other than temperature, (e.g., heart rate, electroencephalogram, blood pressure, and localized radiation dosimetry).

PROGRESS REPORT

A new system to measure and telemeter body temperatures has been developed. This system includes a surgically implantable telemeter and suitable receiving, signal processing, and recording equipment. The implant contains a temperature transducer and its associated circuitry, a transmitter, and a silver oxide cell which provides power to operate the implant for about 18 weeks. As prepared for implantation, the thermal response time constant of this unit is about 10 sec. Temperature can be determined to an absolute accuracy of $\pm 0.05^\circ\text{C}$ over the range of 32–42°C. The system is designed for unattended operation and for easy expansion to multiple parameter measurements through the use of frequency-division multiplexing.

Four transmitters have been built that are designated BT-1, BT-2, BT-3, and BT-4, in chronologic order. Although similar in most respects, each transmitter contained certain changes in design which were indicated by experience gained during the operation of its predecessors. The performance characteristics given above pertain to BT-4. The details of equipment design, calibration and operation will be presented elsewhere.⁽¹⁾

Each of the transmitter designs was surgically implanted within the abdominal cavity of a beagle dog. One transmitter was implanted on two separate occasions. Operating life, after implantation, ranged up to 6 weeks. In 4 cases, the transmitters failed because moisture penetrated into the unit. Improved methods of encapsulation have been designed but have not, as yet, been tested.

The results from recording of body temperature in dogs maintained under a variety of environmental conditions show that temperature responds to three basic factors: (1) excitement related to handling and local disturbances, (2) physical activity of the dog, and (3) ambient temperature and humidity.

In last year's Annual Report⁽²⁾ we indicated that a 24-hr body temperature rhythm exists in the beagle dog. Temperature records over many days from dogs (including the dog reported on previously) in controlled environments gave no indication of a diurnal rhythm. Weather records, obtained from the ANL Meteorology Group, show that changes in body temperature recorded by the first implant correlate with changes in ambient temperature. The transmitter (BT-1) was implanted during the summer, when the days were reaching a maximum ambient temperature of 33°C and a minimum of 18°C. The results reported below confirm the relationship between a dog's temperature and the ambient temperature.

Normal Ambient Temperatures

Three dogs had an average normal body temperature of 37.65°C, based upon many days of continuous temperature recordings. This was appreciably lower than the 38.4°C generally regarded as normal for the dog.

One of the three dogs was placed in three different environments at approximately the same ambient temperature (24–28°C). These areas were the kennel, a metabolism cage in the kennel, and an environmental test chamber isolated from the kennel.

In order of presentation, these changes in the environment were necessarily accompanied by decreased physical activity and reduced psychologic stimulation. As shown in Figure 206, these changes were associated with a progressive stabilization of body temperature.

Above Normal Ambient Temperatures

One animal was isolated in a controlled environment room and acclimated to the conditions of 25°C and $45\% \pm 5$ relative humidity over a period of 1 days. The ambient temperature was then elevated to 40°C with the same relative humidity. This dog's body temperature appeared to depend on the ambient

FIG. 206.—Continuous body temperature response of a dog in 3 different environments of the same ambient temperature (24–28°C). X indicates temperature rise due to cage cleaning. Upper curve, environmental test chamber; middle curve, metabolism cage; lower curve, kennel.

FIG. 207.—Body temperature response of a dog upon being exposed to and removed from a heating load of 40°C and 45% R.H. The dog was continuously exposed to this heat for 6 days. Solid line, body temperature; dashed line, environmental temperature.

FIG. 208.—Body temperature responses of a dog upon being exposed to low environmental temperatures. The average temperature for each day is given to the right of the chart.

FIG. 209.—Continuous recording of a dog's body temperature upon being exposed to low environmental temperatures. X indicates temperature rise due to excitement associated with cage cleaning.

temperature, for it followed the rise of the ambient temperature closely (see Figure 207). It was kept at 40°C for 6 days, and during this entire period its body temperature was approximately that of the environment. Toward the end of the period, the dog's body temperature tended to exceed the ambient temperature, and on one occasion it reached 41.45°C. When the ambient temperature was lowered to 25°C, the dog's body temperature quickly followed, as can be seen in Figure 207. Its body temperature fell until it reached 36.0°C, fluctuated below normal for several hours, and slowly returned to normal.

Below Normal Ambient Temperatures

During the winter, a dog was housed during the daytime in a metabolism cage in the kennel area exterior to the building. Outside temperatures as low as -8°C were recorded during this time. Upon exposure, the dog's body temperature was observed to fall and to reach values which were directly related to the ambient temperature. This is illustrated in Figure 208.

As shown in Figure 209, this fall in body temperature in this dog was not compulsory, since excitement, related to handling and recording of its rectal temperature, produced an immediate rise.

As further shown in Figure 209, body temperature did not remain at reduced values when the dog was exposed to outside conditions for 24 hr at a time. Since the environment was not rigidly controlled, however, it is impossible to determine the reasons for this behavior.

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THYROIDITIS IN A CLOSED COLONY OF BEAGLE DOGS

Thomas E. Fritz, William P. Norris, and Ruth C. Zeman

PURPOSE AND METHODS

Histologic examination of thyroid glands from beagle dogs in experiments investigating the response of this gland to localized X-irradiation⁽¹⁾ revealed several cases of thyroiditis. This disease is infrequently reported in the dog and rarely^(2, 3) in the beagle. Because a relationship between thyroiditis and the experimental procedure used in this study was not defined, a survey was made of thyroids in these dogs to determine the incidence of thyroiditis in this colony. This survey included microscopic examination of thyroid specimens collected from normal dogs in this colony. Since a similar disease in man, lymphocytic thyroiditis, or Hashimoto's disease, is interpreted as an autoimmune reaction related to an

inherited defect in the follicular basement membrane,^(4, 5) a preliminary evaluation was made of the hereditary background of each of the affected dogs. It was thought that this survey would be particularly useful in clarifying this relationship since a large portion of the colony is inbred.

PROGRESS REPORT

Specimens of thyroid glands from 108 untreated, nonexperimental dogs (e.g., culled stock animals, control animals, and old breeders) and from 137 experimental dogs (e.g., dogs either injected with radionuclides or given whole-body irradiation) were examined. There were 16 cases of thyroiditis in the nonexperimental group and 14 cases in the experi-

FIG. 210.—Lymphoid nodules in thyroid gland. Hematoxylin and eosin stain

FIG. 211.—Lymphoid nodule showing germinal center in an area of small follicles with scant colloid. Hematoxylin and eosin stain.

mental group. The microscopic features of the disease were similar in all cases, with some differences in the youngest animals (birth to 2 weeks) in the nonexperimental group. Generally, the reaction was the same as that described by Tucker⁽²⁾ and Mawdesley-Thomas and Jolly,⁽³⁾ including nodular and diffuse infiltration of the gland by lymphocytes, plasma cells, macrophages, and, in a few cases, neutrophils and multinucleated giant cells. Lymphoid nodules with germinal centers were a particularly prominent feature of the cellular response. The thyroid follicles showed varying degrees of compression, atrophy, degeneration, and necrosis, depending on the severity of the reaction. There were also numerous small follicles, acini, cords, and sheets of epithelial cells, which differed from normal epithelial cells. The cells were large with a pale-staining, foamy or slightly vacuo-

lated cytoplasm. Figures 210 to 213 illustrate some of the more prominent features of this disease.

Examination of the pedigrees of the affected animals revealed several cases of thyroiditis among littermates and siblings, with a particularly high incidence in one portion of the colony that has been developed toward an inbred line (A line). Using a computer program to determine the ancestral composition of each dog^(6, 7) from which thyroids were obtained, the dogs were divided into four groups. Table 93 shows the incidence of thyroiditis as related to the ancestral composition of the animals. The data in this table show that dogs with an A-line genetic background have a higher incidence of thyroiditis than the other dogs. This supports the hypothesis of a hereditary basis for the development of thyroiditis.

Future plans to evaluate the incidence of this disease

FIG. 212.—Giant cell, lymphocytes and plasma cells surrounding a degenerating follicle. Hematoxylin and eosin stain

and its possible hereditary basis include the development of serologic methods to demonstrate circulating antibodies to thyroglobulin. Such procedures, used previously,^(4, 8) provide a nondestructive method for detecting diseased animals.

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FIG. 213.—Several clusters of parafollicular cells infiltrated with lymphocytes and plasma cells. Hematoxylin and eosin stain

TABLE 93. DISTRIBUTION OF CASES OF THYROIDITIS
ACCORDING TO GENETIC COMPOSITION OF THE
ANIMALS

	Line A ^(a)	Line B ^(a)	Other than A or B ^(a)	Un- known ^(a)	Total ^(b)
Nonexperimental	14/63 (22%)	8/71 (11%)	1/31 (3%)	8/80 (10%)	16/108 (15%)
Experimental	11/73 (15%)	6/87 (7%)	3/34 (9%)	6/107 (6%)	14/137 (10%)

^(a) Number of dogs with thyroiditis with ancestors of this line/total number of dogs examined with ancestors of this line.

^(b) Number of dogs with thyroiditis/total number of dogs examined.

EFFECT OF RESTRICTED IODIDE INTAKE ON THE STRUCTURE AND FUNCTION OF THE THYROID GLAND OF THE BEAGLE

James A. Taylor, Thomas E. Fritz, William P. Norris, and Norbert D. Kretz

PURPOSE AND METHODS

Studies of thyroid ^{131}I uptake and biological half-life of trapped radioiodide in dogs have shown wide variations.⁽¹⁻³⁾ Iodide-deficient diets are reported to be goiterogenic in dogs,⁽⁴⁾ and there are recommendations on supplementation of iodide to prevent deficiency.⁽⁵⁾ There is, however, no information regarding optimum dietary iodide levels in relation to their effects on the gland.

Studies of thyroid physiology in the dog are further complicated by the fact that commercial diets may fluctuate greatly in maximum iodide content, as minimum concentrations are generally the only consideration in formulation of diets.

In the previous section of this report, a study of the effects of localized X irradiation on the surgically isolated thyroid gland of the beagle dog is described. It was stated that the normal thyroid gland of the beagle became unexpectedly hyperplastic when the animals' dietary iodide intake was limited to 50–75 $\mu\text{g}/\text{day}$.

This report describes the progressive change in function and histologic appearance of the untreated thyroids of the dogs in this experiment. They are compared to the thyroids of normal, untreated dogs on the conventional diet used in this Laboratory.

One control animal was sacrificed after approximately 4.0, 6.0, 9.0, 12.0, and 15 months on the low-iodide diet to obtain both gross weights and specimens for histopathologic examination. In addition, thyroid glands were obtained from the experimental animals that were thyroidectomized. Tissue specimens of animals on the conventional diet were obtained from colony stock sacrificed at 11 months of age. Necropsies were performed on all sacrificed animals, and their thyroids were weighed and fixed for preparation of histologic sections, using hematoxylin and eosin.

PROGRESS REPORT

The data pertinent to the change in function of the thyroid gland in relation to time on a restricted iodide intake are summarized in Table 94. These data are primarily a recapitulation of data from control animals from the previous report. After 50 days, ^{131}I uptake increased significantly and maintained a slow but noticeable rise through 350 days on the low-iodide diet. Increased uptake values, be-

tween 100 and 350 days, are significant here at the $p = 0.01$ level. In addition, the time required for maximum uptake decreased in relation to time on the low-iodide diet until after 170 days, when maximum uptake was noted at the first measurement (24 hr) after radioiodine administration.

Iodine-131 was released from the thyroid with a biologic half-time which also decreased significantly in relation to time on the low-iodide diet. Based on previous data of Toft et al.,⁽⁶⁾ there was an apparent decrease of biologic $T_{1/2}$ (or increased rate of thyroid hormone release) noted over the next 260 days to a value of 4.0 days. The next measurements, taken at 350 and 440 days, showed a change in this trend. There was a marked increase in biologic $T_{1/2}$ to 26.0 and 37.5 days, respectively.

Histopathologic examination showed progressive and significant alterations in cellular morphology and structural architecture of the thyroid gland throughout this period of restricted iodide intake. Initial changes consisted of loss of colloid from the follicular lumen to the extent that little or no colloid was observed in thyroid tissue removed after 100 days. Progressively severe cellular and follicular hyperplasia and hypertrophy were observed from 100 to 260 days (Figures 214 and 215). The hyperplasia resulted in marked enlargement of follicles and marked infolding of the epithelium.

TABLE 94. THYROID UPTAKE AND RELEASE OF IODINE-131

No. of animals	Time on low-iodine diet, days	Max. thyroid uptake ^{131}I , %	Time to peak ^{131}I uptake, days	Biol. (a) $T_{1/2}$, days	Total thyroid weight, g
8 ^(b)	0	11	2–3	10–15	0.90 ^(e)
6	50	24	2.1	8.2	—
—	75				0.87 ^(d)
6	100	47	1.4	7.2	1.90 ^(e)
5	170	54	1.2	5.8	2.65 ^(e)
4	260	60	1.0	4.0	3.05 ^(e)
3	350	65	1.0	26.0	1.57 ^(e)
2	440	66	1.0	37.5	2.95 ^(e)

(a) Biological half-time of disappearance of activity from the thyroid measured from 1 to 12 days post-injection.

(b) Data calculated from work of Toft et al.⁽⁶⁾ conducted with similar beagle stock in this laboratory.

(c) These weights are from one dog sacrificed at each interval.

(d) Mean value from 6 totally thyroidectomized dogs.

(e) Mean value from 10 untreated dogs 11 months of age.

FIG. 214.—Thyroid gland of dog on commercial dog food

An increase in thyroid weight was seen by 100 days after initiation of the low-iodide dietary regimen (Table 94), and further increases were seen at still later times.

Throughout the course of this study, there were no clinical signs resembling myxedema or hypothyroidism in these dogs. Hemograms indicated no significant change in red cell mass, as might be apparent with hypothyroid dogs, nor were there any significant changes in blood calcium. When the low-iodide diet was begun, serum cholesterol levels rose abruptly from about 100 mg % on the conventional diet to about 200 mg %. A slight decrease in PBI levels was noted, although they were not significantly lower than values seen under normal feeding conditions. No free iodide was found in the blood of dogs on limited iodide diets, whereas amounts from

4–10 μg % were found in beagle dogs maintained on the conventional Rockland dog diet.

CONCLUSIONS

These studies show that when iodide intake is restricted to 50–75 $\mu\text{g}/\text{day}$ in the adult beagle, the changes in the histologic features of the thyroid gland are initially those of a marked hyperplasia and an associated increase in ^{131}I uptake and decrease in biologic $T_{1/2}$. These histologic changes are generally regarded as indicative of a deficiency in dietary iodide. After 350 days, however, there was a dramatic change in ^{131}I uptake and associated biologic $T_{1/2}$, which correlate with a more normal though still hyperplastic, appearance, of the gland. These changes are provocative and suggest an adapta-

FIG. 215.—Thyroid gland of dog on restricted iodide intake for 100 days. There is hyperplasia and hypertrophy of the epithelium and little colloid in the follicles.

tion or change in thyroid activity that merits more careful investigation.

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COLONY STATUS: BREEDING PROGRAM EVALUATION

Calvin M. Poole, William P. Norris, and Robert J. Flynn

PURPOSE AND METHODS

The beagle breeding program, its purpose, and the origin of its breeding stock nucleus have been described.⁽¹⁾ The entire colony has been maintained since 1960 as a closed colony; that is, no new acquisitions have been made from outside this Laboratory. The primary purpose of the closed-colony principle was to minimize the introduction of clinical diseases into the colony. This has been remarkably successful, as evidenced by the lack of the common infectious diseases of dogs (e.g., canine distemper, canine hepatitis) and the low incidence of parasites.

The breeding program has been conducted so that it minimizes inbreeding in a portion of the colony, which is referred to as outbred. In addition, a deliberate effort to develop more homozygous animals has been made in which inbreeding has been practiced. This has resulted in the development of two ancestral lines which are designated as Alabama (A-line) and Blond (B-line). Within these lines, the individual matings have ranged from brother × sister mating to the pairing of more distant relatives within the line. The intent in each case was to produce acceptable offspring with increasingly higher inbreeding coefficients. The method of calculating inbreeding coefficients has been described elsewhere.⁽²⁾

In order to evaluate the efficacy of the breeding program as practiced, the past breeding histories were reviewed, determinations were made of litter size, number weaned per litter, number stillborn per litter, neonatal deaths (birth to 7 days) per litter, deaths from 7 days to weaning per litter, and the conception rate. While tabulations were made for the entire colony, no matings of experimental dogs were included. To compare the animals, the data were subdivided into outbred, A-line, and B-line matings. No attempt was made to relate reproductive efficiency to inbreeding coefficients because the data were insufficient to warrant such an evaluation at this time.

PROGRESS REPORT

Breeding data relevant to litter size, number of dogs weaned, number stillborn, neonatal deaths, and deaths from 7 days to weaning are summarized in Table 95. These data were compiled from 1959 to May, 1967. They were also plotted as a function of chronological time for the outbred group, the A-line, and total colony (Figures 216 to 218). The data avail-

TABLE 95. COMPARISON OF LITTER SIZE AND PUP SURVIVAL BETWEEN OUTBRED DOGS AND TWO LINES OF PARTIALLY INBRED DOGS

Strain..... No. of litters.....	A-line 64	B-line 22	Outbred 171	Total 257
Mean litter size	5.33	3.54	5.17	5.07
Mean number weaned/ litter	2.72 (51%)	1.23 (35%)	3.98 (77%)	3.43 (68%)
Mean number stillborn/ litter	0.84 (16%)	0.86 (24%)	0.51 (10%)	0.62 (12%)
Mean number of deaths, 0-7 days	1.47 (28%)	0.86 (24%)	0.46 (9%)	0.75 (15%)
Mean number of deaths, 7 days to weaning	0.30 (6%)	0.55 (15%)	0.22 (4%)	0.27 (5%)

FIG. 216.—Chronological summary of pup mortality and survival in the outbred group.

able for the B-line were insufficient to be examined in this manner. The mean litter size in the outbred group and A-line dogs is quite comparable to that reported in other colonies.^(3, 4) The litter size for the B-line appears to be significantly smaller. The out-

tanding feature of this information is the differences in weaning percentage: the outbred group again compares closely with that reported elsewhere, while the two inbred lines have appreciably lower overall weaning percentages. In general, these weaning percentages show a progressive decrease, and these decreases appear to be correlated to increased losses from birth to 7 days. The cause of the increase in neonatal losses is not readily explained; the majority of the losses appear to be caused by bacterial infections which are not easily related to management practices.

In order to characterize further the breeding efficiency of this colony, the conception rate for the period June, 1963 to June, 1967 was determined, as is shown in Table 96. This is expressed as a percentage of matings that led to full-term pregnancies. It includes both natural matings and those accomplished by artificial insemination. Artificial insemination is used when it becomes apparent that a natural mating will not occur. The relatively *low* conception rate for the A-line is the outstanding feature of these data. To characterize, at least partially, the cause of this failure to conceive, semen evaluation was undertaken. The results of this are reported elsewhere in this report. It was demonstrated, however, that the A-line dogs have much lower sperm production than outbred

FIG. 217.—Chronological summary of pup mortality and survival in the partially inbred A-line.

FIG. 218.—Chronological summary of pup mortality and survival in the entire colony.

TABLE 96. A COMPARISON OF CONCEPTION RATES IN OUTBRED DOGS AND TWO LINES OF PARTIALLY INBRED DOGS (JUNE 1963-JUNE 1967)

Strain..... No. of females	Percent of breedings that resulted in full-term pregnancies			
	A-line 32	B-line 6	Outbred 28	Total 66
Natural breedings	59.5	100	86.4	74.7
Artificial breedings	55.6	50	45.0	48.5
All breedings (natural and artificial)	56.4	88.2	83.3	70.4

males. This readily accounts for the lower conception rate in this line.

CONCLUSIONS

Although the data presented are insufficient for detailed analysis, they suggest certain general trends. They show that the B-line produces smaller litters.

While the A-line has a normal litter size, it has a low weaning percentage. It also has a low conception rate, which is partially attributable to the reduced spermatogenesis identified in this line. Reproduction in the outbred group is very similar to reproduction reported for other beagle colonies^(3, 4) in which inbreeding has been minimized.

The only effect directly attributed to inbreeding per se is the lower conception rate and reduced spermatogenesis.

genesis observed in the A-line. This is not surprising, since such occurrences are frequently seen in other species when extensive inbreeding has been practiced.⁽⁵⁾ That reproductive ability in the inbred lines of these dogs is not worse is highly encouraging, since the need for an inbred dog for critically controlled work is becoming increasingly apparent.

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COLONY STATUS: STUDIES ON ORGAN WEIGHTS IN BEAGLE DOGS

Thomas E. Fritz, William P. Norris, and Ruth C. Zeman

PURPOSE AND METHODS

Organ weights are commonly used as a method for evaluating animal health or disease in studies of acute or long-term toxicity. Changes in organ weights help to identify affected sites in studies where experience with organs likely to be affected is lacking. Information on organ weights of dogs is available, but is of limited use because of great variations, particularly between breeds, within *Canis familiaris*,⁽¹⁻³⁾ and also because of variations in body size, sex, and age.⁽⁴⁻⁶⁾ Although size, as measured by body weight, is usually considered in analyses of organ weights, nutritional status, state of obesity, or conformation of the animal are rarely considered. Relative organ weights (organ weight/body weight) have been used to reduce variability,⁽⁷⁾ but they are also complicated by a number of factors,⁽⁸⁾ and must be examined by appropriate statistical methods.⁽⁹⁾

The study reported here was initiated to provide a more definitive description of organ weights in beagles used in toxicity experiments, many of which are highly inbred, and also to provide a basis for assessing the effects of experimental procedures on these animals. It is hoped that it will also establish a criterion for defining an "acceptable" or "standard" animal and assist in the breeding program used in this colony.

In an earlier report⁽¹⁰⁾ we described the preliminary analysis of organ weights by regression methods to determine relationships between organ weights and total body weight and between organ weights, when these are expressed as a fraction of body weight, and total body weight.

PROGRESS REPORT

During the past year, an additional method for evaluating organ weights from nonexperimental adult dogs has been employed. Because of the phenotypic

TABLE 97. RATIO OF ORGAN WEIGHTS TO FEMUR WEIGHTS IN UNTREATED ADULT BEAGLES

Organ	Mean of 39 anesthetized, exsanguinated animals	Standard deviation	Mean of 12 anesthetized animals	Standard deviation	Significance (Student's <i>t</i> test)
Liver	9.47	3.04	9.17	1.81	20
Heart	2.57	0.41	2.80	0.48	80
Stomach	2.57	0.55	2.83	0.42	80
Small intestine	8.20	1.39	8.89	1.26	80
Large intestine	2.09	0.40	2.39	0.53	95
Thyroid	0.03	0.01	0.02	0.003	70
Adrenal	0.04	0.01	0.04	0.01	70
Ovaries	0.05 ^(b)	0.02	0.07 ^(d)	0.05	80
Testes	0.46 ^(c)	0.16	0.69 ^(e)	0.28	98
Spleen ^(a)	24.21	6.57	64.83	17.81	999
Brain ^(a)	81.11	7.66	77.83	6.47	80
Lungs	2.25	0.32	2.51	0.53	95
Kidneys	1.59	0.26	1.97	0.35	999
Pancreas	0.72	0.18	0.79	0.16	80
Pituitary	0.002	0.0006	0.002	0.0005	80
Prostate	0.23 ^(c)	0.10	0.38 ^(e)	0.19	98
Eyes ^(a)	11.31	0.96	11.66	0.92	70

(a) Actual weight.

(b) 20 Females.

(c) 18 Males.

(d) 5 Females.

(e) 6 Males.

differences between the animals, it was deemed necessary to find a method to correct the organ weight of an animal in terms of its size, conformation, or obesity. Examinations of the femur weight showed that this organ is a constant fraction of the skeletal weight; the skeleton is believed to vary minimally in relation to animal obesity, activity, and most disease. Organ weights, therefore, were normalized by computing the specific organ weight to its femur weight ratio. The weights of the eyes, spleen, and brain, which

have previously been shown to be essentially independent of body size,⁽¹⁰⁾ were not normalized.

Because many experimental dogs are allowed to die before a necropsy examination is conducted, and other experimental dogs, and most control dogs are sacrificed by exsanguination, it is also necessary to determine the effects of exsanguination on their organ weights.

Table 97 lists, and compares, the computed ratios of organ/femur weights for untreated adult beagles which were anesthetized with barbiturates and exsanguinated, and those which were killed by an excess of barbiturates, but not exsanguinated. The weights of six organs—large intestine, testes, spleen, lungs, kidneys, and prostate—were significantly different at the $p > 0.05$ level. The highly significant difference between the two ratios for the spleen is due to engorgement of the spleen as response to barbiturates. The highly significant difference in ratios for the kidneys over other organs was not anticipated.

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ESTIMATION OF SEMEN QUALITY IN OUTBRED AND PARTIALLY INBRED BEAGLES

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PURPOSE AND METHODS

In an analysis of the beagle breeding program, found elsewhere in this report,⁽¹⁾ a lower conception rate was described for the inbred group designated as A-line than for the outbred group. Also, during the course of performing artificial insemination, aspermic semen was occasionally observed in some males of the A-line. Since this is a closed colony, entirely dependent upon reproduction from within, a reduced conception rate is important.

Infertility problems can reside in either males or females, or in both. However, because the male genital organs can be easily examined and semen evaluation is readily accomplished, the males were selected for this study. Also, there is evidence that the genetic makeup of the male may determine his fertility and potency.⁽²⁾

Of 30 male beagles involved in this study, 15 were from the inbred A-line and 15 were from the outbred line (minimal inbreeding). Wright's inbreeding coefficients were calculated for all animals by a method described previously.⁽³⁾ All animals were anatomically normal, 2 to 6 years of age, and in either stock

or breeder classifications. None of the animals used were littermates, although several were siblings. All of the animals used have been maintained since birth under the same environmental conditions. To minimize the possibility of the effect of a nongenital secondary disease process, all dogs were given a complete physical examination when the study began as an adjunct to their routine physical examinations. Their clinical records were also examined for evidence of any previous history that would have influenced reproductive ability.

Each animal was ejaculated for a semen examination at least three times at weekly intervals. This schedule avoids an evaluation based on one sample, which can be very misleading, since it has been observed that the first ejaculate may be of poor quality from animals that have not ejaculated for long periods of time. Each ejaculate was examined as soon as possible after it was collected by using a standard battery of semen tests.⁽⁴⁾ Physical observations of volume and color were immediately recorded, and a drop of semen transferred to a warm microscope slide was observed to grade sperm motility. Motility was described as follows: 0, immotile; I, weak rotary movements; II, rotary and progressive motion, with no waves or eddies; III, progressive rapid movement, with

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TABLE 98. SPERM CELL COUNTS FOR A-LINE AND OUTBRED GROUPS

Dog No.	Inbreeding coefficient	Number of cells/ml ($\times 10^4$)			
		Ejaculate			
		1st	2nd	3rd	4th
A-line group					
376	0.448	401	242	182	195
531	0.448	4	0	2	3
651	0.448	46	121	48	88
872	0.547	48	59	164	—
879	0.547	0	0	0	—
1004	0.547	45	76	18	—
469	0.555	137	117	328	100
497	0.555	0	11	6	7
499	0.555	0	0	4	4
538	0.555	128	118	101	222
541	0.555	34	135	28	18
591	0.555	39	80	122	146
663	0.555	0	0	0	0
749	0.631	0	0	0	0
715	0.635	95	56	62	—
Outbred group					
389	0.0	465	539	633	—
584	0.0	598	290	631	—
735	0.0	826	882	851	—
743	0.0	459	304	387	—
780	0.0	307	56	432	—
700	0.015	359	372	370	—
720	0.015	0	0	0	—
403	0.021	999	1131	904	993
794	0.023	417	591	591	—
885	0.056	260	891	641	—
998	0.081	967	1031	964	—
1066	0.081	218	613	478	—
1070	0.081	59	1000	862	—
755	0.083	179	350	385	—
1032	0.259	918	779	982	—

TABLE 99. FREQUENCY DISTRIBUTION OF SPERM CELL CONCENTRATION AND SEMEN MOTILITY GRADING

	Mobility					Number of sperm/ml		
	0	I	II	III	IV	$< 10^4$	$10^4 - 10^6$	$> 10^6$
A-line group (15 dogs, 56 ejaculates)	27	8	17	4	0	14	25	17
Outbred group (15 dogs, 46 ejaculates)	3	1	6	14	22	3	2	41

slowly moving waves; IV, vigorous, progressive movement, with rapid and abruptly forming waves and eddies.⁽⁴⁾

Duplicate slides were prepared for staining. One

was stained using the eosin-nigrosin method.⁽⁵⁾ With this technique, the dead spermatozoa stain red and the live spermatozoa remain colorless against the dark nigrosin background. The other was fixed in ethyl alcohol and stained according to the procedure of Casaretti.⁽⁶⁾

Morphologic variations of the stained spermatozoa were determined by counting the number of abnormal cells per 100 cells examined. Primary abnormalities were noted separately, since these occur during spermatogenesis and may be the result of genetic defects in the cell.⁽³⁾

The concentration of sperm in the ejaculate was determined by using a hemocytometer and a red blood cell pipette in a technique similar to that used in red blood cell counting, except that 10% sodium bicarbonate was used as the diluent. The hydrogen ion concentration of the undiluted semen was measured with a standard laboratory pH-meter.

PROGRESS REPORT

The sperm cell concentration in each ejaculate studied is shown in Table 98. The mean cell count for the A-line dogs was $68.9 (\times 10^4)$, and the count for outbred dogs was $564.4 (\times 10^4)$. The motility grading was directly related to sperm counts, and frequency distribution of both is shown in Table 99.

Semen color, which varied from water clear to creamy white, was directly related to sperm cell concentration. The volume varied from 1.2 to 5.0 ml in the A-line and from 2.2 to 5.4 ml in the outbred group. All volumes are in the range of normal values. pH values for these ejaculates are also considered to be in the normal range.

Sperm morphology examinations showed means of 15.8% primary abnormalities for the A-line group and 3.7% primary abnormalities for the outbred group.

CONCLUSIONS

Although the sperm counts are not directly related to the inbreeding coefficients, the more highly inbred A-line has lower counts than does the outbred group. We interpreted this as a reduced spermatogenesis and must conclude that it is partially responsible for the reduced conception rate obtained from this line.

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STUDIES ON SPONTANEOUS DISEASE AND PATHOLOGY IN THE EXPERIMENTAL BEAGLE COLONY

Thomas E. Fritz, Ruth C. Zeman, and William P. Norris

PURPOSE AND METHODS

Because the beagle dog in our colony must be critically defined, data have been collected on the natural occurrence of anomalies, (e.g., cryptorchidism), disease, (e.g., thyroiditis), and neoplasms, (e.g., osteogenic sarcomas). To store, retrieve and interpret these data, record systems utilizing punch cards and computer tape programs were developed.^(1, 2) These data-handling programs include a coding system for recording descriptive pathology, organ weights, and inbreeding coefficients.

In a previous report,⁽³⁾ we listed the anomalies and neoplasms observed to that time, a period of over 6 years. During the last year, we have updated the list of anomalies. Only one new tumor was seen, a primary malignancy of the stomach that has not been classified.

Since a large portion of the colony is highly inbred, it seemed desirable to study the effects of inbreeding on the incidence of anomalies, as inbreeding is generally thought to result in increased anatomical defects. A preliminary correlation between the incidence of anomalies and the inbreeding coefficient has been made in 1015 dead dogs. They were arbitrarily divided in two groups, those with an inbreeding coefficient below 0.3 and those above 0.3. These two groups were further divided into dogs that had reached weaning age (42 days) and those that had not been weaned. This was done because cryptorchidism, one of the most common anomalies, was difficult to detect in young male dogs; the testicles are not normally in the scrotal position until several weeks after male beagles are born.

PROGRESS REPORT

Table 100 lists the anomalies observed in 916 animals examined through October 1, 1967. These animals include those used for all purposes in the colony breeding, control, radiation experiment, diet, etc.) and those having both spontaneous and induced diseases. No anomaly is included in this list that could have been directly induced by the experimental pro-

TABLE 100. ANOMALIES PRESENT IN 917 NECROPSIED BEAGLE DOGS

Type of anomaly	Occurrence			
	Total	Still-born	Suck-ling	Adult
Hydrocephalus	23	1	4	18
Cryptorchid (43 dogs) ^(a)	47			43
Unilateral renal agenesis	7	3	2	2
Abnormal renal morphology	4			
retained fetal lobulation		1		
hypoplasia and spheroidal		2		
medullary hypoplasia			1	
Hydronephrosis	1	1		
Atresia of ureter	1			1
Agenesis of ureter	2	1	1	
Hypoplasia of small intestine	5	4	1	
Diverticuli of the small intestine (Meckel's diverticulum)	5			5
Redundant small intestine	1			1
Cecal agenesis	1	1		
Redundant cecum	1		1	
Small peritoneal cavity	1	1		
Omphalocele	3		3	
Incomplete cutaneous covering of skull	1	1		
Agenesis of pinnae	1		1	
Plagiocephaly	1		1	
Fusion of maxillomandibular joint	1	1		
Cleft palate	2	1	1	
Harelip	2	1	1	
Vertebral defect ^(b)	4	1	2	1
Congenital diaphragmatic hernia	2	2		
Persistent thyroid isthmus	26	3	9	14
Thyroglossal duct cyst	1			1
Ectopic thyroid	1			1
Heart or blood vessel defect (patent foramen ovale, patent ductus arteriosus, ventricular system defect, nonpatent pulmonary artery)	13	6	6	1
Misalignment of tracheal rings	3			3
	160			

^(a) Bilateral cryptorchids counted as 2 anomalies.

^(b) Enlarged right anterior facet of 3rd cervical vertebra, sharp dorsal flexure of the cervical vertebrae, and scoliosis of the thoracic vertebrae (2 cases).

TABLE 101. CORRELATION BETWEEN INCIDENCE OF ANOMALIES AND INBREEDING COEFFICIENT IN 1015 DEAD DOGS

Age	Inbreeding coefficient	Total dogs	Total anomalies ^(a)	Percent			
				Hydrocephalus	Cryptorchid	Other	Total
Dogs weaned (42 days or older)	0.00-0.30	406	48	3.7	6.7	1.4	11.8
Dogs weaned (42 days or older)	0.31-0.70	82	27	3.7	19.5	9.7	32.9
Dogs not weaned (less than 42 days)	0.00-0.30	299	16	1.0	(6.7) ^(b)	4.4	5.4 (12.1) ^(c)
Dogs not weaned (less than 42 days)	0.31-0.70	228	34	0.9	(19.5) ^(b)	14.0	14.9 (34.4) ^(c)

^(a) Uncorrected for animals not examined because of cannibalism, postmortem decomposition, or transfer to other projects.

^(b) Percent cryptorchid predicted based on incidence in the weaned age group with the same range of inbreeding coefficients.

^(c) Percent total anomalies predicted based on adding percent cryptorchid predicted to total observed.

cedure used to study that animal (radiation, nutrition, and environment).

Table 101 summarizes our preliminary correlation between the incidence of anomalies and the inbreeding coefficient. In order to interpret better the influence of two of the most common anomalies—hydrocephalus and cryptorchidism—they have been considered individually.

The data in Table 101 indicate that in both age groups the dogs with an inbreeding coefficient greater than 0.3 had 2.8 times as many total anomalies as those with a coefficient less than 0.3. The incidence of cryptorchidism in the weaned age group, similarly, was higher in the dogs with the higher inbreeding coefficient. The incidence of hydrocephalus was the same, regardless of the age or the inbreeding coefficient.

The percentage of total anomalies was smaller, however, in the dogs less than 42 days of age, obviously because cryptorchidism is not recognized in

this age group. When the predicted percentage of cryptorchids, as derived from the percentage observed in the older group, is added to that observed, the total closely approximates that observed in the older group.

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THE PROGRESS OF PERIODONTAL DISEASE IN THE AGING BEAGLE HOUND

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PURPOSES AND METHODS

The purpose of the present study is to achieve a more objective clarification of the characteristics of the periodontium in the aging unirradiated beagle hound. This report deals only with the clinical observations of oral health.

Periodontal disease is enzootic in the beagles bred and maintained in our laboratory when no specific measures are taken to control excessive accumulation of calculus on the teeth.⁽¹⁾ The colony was started

in 1960 with mature dogs as foundation breeding stock, and provisions were made at that time to study dental disease because periodontitis had been reported in beagles in a similar environment.⁽²⁾ It was concluded that routine manual removal of tartar and calculus at four-month intervals would prevent periodontal disease, and this means of prophylaxis for the disease was initiated in late 1963. The prophylactic treatment permitted the study of radiation damage, uncomplicated by the lesions of periodontal diseases when treatment was initiated at 8 to 12 months of age. The foundation stock dogs and the early experimental dogs, however, had already suffered vary-

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...g degrees of irreversible injury. Since the changes due to the periodontal disease simulate and mask those of radiation, it was deemed necessary to study the naturally occurring disease *per se*. The information from this study could then be used to evaluate changes where radiation damage and periodontal disease were concurrent.

Prophylaxis in the form of routine scaling of teeth was withheld from a group of 12 dogs, equally divided as to sex, born in 1960 and 1961 (see Table 102). All dogs in the colony live in a controlled environment, including receiving a commercial dog food in expanded pellet form. Each of the 12 untreated dogs has received the commercial diet; 2 males and 2 females, however, were given a dietary supplement of $\frac{1}{3}$ raw oxtail per dog, offered once each week for the past 27 months. Oxtail has been used successfully to prevent excessive calculus accumulation in a beagle colony in a somewhat different controlled environment.⁽³⁾

The incidence and pattern of calculus deposition and gingival inflammation in our experimental group was reported previously.^(1, 4) Observations were made at approximately 270 days, 420 to 450 days, and 570 to 600 days. The dogs now average approximately 2300 days of age and their present status is evaluated for this report; the authors know of no study of periodontal disease in dogs covering as long a period of clinical observation.

The following criteria were developed at the onset of the study to allow valid interpretations and comparisons to be made as part of a linear or longitudinal study.⁽⁴⁾

Examination Procedures

1. Clinical examination: Oral cavity examination and charting specifically for evaluation of
 - a. Tooth cleanliness and calculus
 - b. Gingival tissue
2. Special procedure
 - a. Intraoral photographs (Kodachrome, standardized color-temperature) to record observations objectively

Criteria of Assessment

1. Assessment of tooth cleanliness
 - a. No evidence of stain on labial (buccal) or proximal areas of enamel
 - b. A film or stain evident on labial (buccal) or proximal areas of enamel; accumulation of soft debris not apparent clinically or on colored photographs
 - c. Accumulation of soft debris (not calcified) on

TABLE 102. AGE AND SEX DISTRIBUTION OF EXPERIMENTAL GROUP AT TIME OF OBSERVATIONS

Controlled diet		
Dog No.	Sex	Age, days
210	F	2398
211	F	2398
209	F	2412
218	F	2401
310	M	2194
312	M	2208
317	M	2207
318	M	2207
Controlled diet supplemented with oxtail		
206	F	2413
205	F	2413
304	M	2213
311	M	2208

the labial (buccal) or proximal surfaces in addition to film or stain as described above

- d. Presence of calculus (apparent clinically or in the photographs) in addition to stained plaque and soft debris

- (1) Indicates amount of calculus present about cemento-enamel junction
- (2) Same as above, calculus to mid-crown
- (3) Same as above, calculus above mid-crown to incisal edge

2. Assessment of gingival inflammation. Degree of inflammation:

- 0 = Clinically normal gingiva
- 1+ = Mild inflammation—slight change in color and little change in contour
- 2+ = Moderate inflammation—swelling obvious, glazing, redness (edematous), and chronic inflammation
- 3+ = Severe inflammation—more swelling and redness, possible pocket formation, sup-puration and tissue degeneration

The oral cavities of all dogs in the experimental group were carefully examined, and the previously described items of data were recorded. The examination was performed while the animal was under sodium pentothal anesthesia.

PROGRESS REPORT

Dogs fed only the commercial dog food showed a marked increase in calculus deposition. Clinical observations suggested a definite change in the appearance and character of the deposited calculus at 2300 days of age, as compared to 450 or 600 days; this calculus has a surface roughness and solidity that was

FIG. 219.—Average degree of calculus deposition and gingival inflammation.

not observed previously. Calculus deposition developed from the level of gingival tissue and, in many instances, extended into the subgingival area of the gingival crest. The color of the calculus varied in relation to its proximity to the gingivae. The deposition subgingival, or at the crest of the tissue, was dark and nearly black, indicating that hemorrhage from the gingival tissue had seeped into the calculus deposits. The color of the calculus remains a grayish-brown, apart from the area affected by the hemorrhage.

The overall increase in gingival inflammation is remarkable; changes in color, contour, and consistency of the gingival tissue have been observed. The most advanced changes were localized extreme swelling and redness, pocket formation, suppuration, and tissue degeneration. Both moderate and advanced changes occurred in the 8 dogs that were not given the oxtail diet supplement.

The degree of calculus deposition and gingival inflammation is greater in the maxillary arch than the mandibular arch. Figure 219 demonstrates the aver-

age degree of calculus deposition and gingival inflammation.

The portion of the experimental group which received the oxtail supplement demonstrated a markedly different picture. A film, or stain, and the accumulation of a minor amount of soft debris were present on the maxillary and mandibular premolars and first molars. The maxillary fourth and third premolars showed a narrow line of dark calculus deposited on the buccal surface near, but separated from, the crest of the gingival margin. Mild gingival inflammation, with a slight change in color and contour, was observed about the maxillary and mandibular premolars and first molars.

The maxillary and mandibular canines showed a definite deposition of calculus and gingival inflammation. The calculus deposition extended from the crest of the gingival tissue and subgingivally to the midcrown of the tooth. Gingival inflammation was present only about the maxillary and mandibular canines and was classified as moderate, swelling obvious, glazing, redness (edematous), and chronic inflammation.

CONCLUSIONS

The occurrence of calculus and gingival inflammation was not equal in all animals of approximately equal age, but the pattern becomes more uniform as the animals grow older. Concomitant calculus formation and gingival inflammation, however, represent a general common denominator for this group, which exhibits frank periodontal disease.

The introduction of oxtail, as a supplement to the commercial pelleted diet, has controlled calculus accumulation and gingival inflammation in beagle dogs in our controlled environment.

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RADIATION DOSAGE IN X-RAY EXAMINATIONS OF ORAL STRUCTURES

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PURPOSE AND METHODS

Lithium fluoride microdosimeters are being used to measure both absolute and proportionate X-ray doses to skin, teeth, and other oral structures in dental patients. This pilot study was designed to develop a technique to use these dosimeters in radiography, radiotoxicity studies, and radiotherapy. The microdosimeters consist of 9 mg annealed lithium fluoride loaded into sections of plastic tubing, measuring 1×10 mm in outside dimensions. Each dosimeter is placed in a selected anatomical location to receive an X-ray dose during routine dental radiographic procedures. The dental films are processed to assure adequate exposure in order to produce diagnostic X-ray images. The microdosimeters are read in the manner described by Kastner et al.⁽¹⁾ The purpose of this study is as follows:

Phase I

The objective of this phase of the study was to measure radiation intensities at the skin, buccal fold, and at the source side of the intraoral film in 24 randomly selected patients who presented for routine radiographic surveys at the clinic of the University of Illinois College of Dentistry.

Eight periapical areas, 4 in the maxilla and 4 in the mandible, were selected as the examination regions for radiation measurements. One lithium fluoride dosimeter was placed on the skin, another adjacent to the gingival tissue between the cheek and teeth in the buccal or labial fold, and the third was positioned on the source side of the dental intraoral X-ray film packet. For each exposure, the 3 dosimeters were positioned linear to the center beam. Only one periapical series was measured for each of the 24 patients during their routine radiographic survey. This procedure permitted 3 radiation measurements for each periapical examination, providing a total of 72 measurements.

Phase II

The purpose of this phase was to measure the cumulative radiation exposures at 3 selected anatomic sites, both eyes and the thyroid, during routine radiographic surveys. The routine or complete survey in his case requires 20 intraoral film exposures.

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TABLE 103. ATTENUATION RATIOS OF MEASURED
X RADIATION, 90 kVp, 1.5 MAS, 8" CLOSED
CONE

Area	Skin (100%)	Buccal fold		Film	
	mR*	mR*	%	mR*	%
Maxilla	336	230	68	57	17
Molar	361	179	50	68	19
	344	67	19	27	8
Premolar	392	216	55	51	13
	348	219	63	58	17
	373	214	57	43	12
Cuspid	377	289	77	63	17
	373	299	80	76	20
	324	167	52	54	17
Incisor	329	287	87	96	29
	340	252	74	69	20
	231	116	50	32	14
Mandible	304	130	43	28	9
Molar	352	165	47	97	28
	302	151	50	59	20
Premolar	267	203	76	38	14
	311	—	—	31	10
	332	178	54	96	29
Cuspid	389	266	68	88	23
	445	189	42	47	11
	364	234	64	95	26
Incisor	323	278	86	115	36
	317	241	76	99	31
	378	264	70	123	33
Mean	342	210	61	67	20

* LiF is about 30% more sensitive to these low voltage dental X rays, based on equivalent ⁶⁰Co γ -ray exposure.

Dosimeters were placed on the skin below the lower lids of the right and left eyes and just above the level of the infraorbital ridges. The third dosimeter was placed on the skin over the superior thyroid notch, or laryngeal prominence.

Twelve randomly selected patients, who also presented themselves for routine radiographic surveys at the school clinic, served as the sample group. Three dosimeters per patient for measurement of radiation

TABLE 104. RADIATION LEVELS, 90 kVp, 1.5 MAS,
8" CLOSED CONE

Site	Range, mR*	Mean, mR*
Left orbit	743-1000	855
Right orbit	756-1061	954
Thyroid	67-199	125

* LiF is about 30% more sensitive to these low voltage dental X rays, based on equivalent ^{60}Co γ -ray exposure.

Total exposure time = 1.74 sec for intraoral films.

intensity during each radiographic survey provided a total of 36 readings.

PROGRESS REPORT

The radiation intensities recorded for phase I of this study are shown in Table 103.

The radiation intensities recorded for phase II of this study are shown in Table 104.

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CONTINUING STUDIES OF ^{137}Cs IN FISH FROM THE RED LAKES, MINNESOTA

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PURPOSE AND METHODS

The purpose of this investigation⁽¹⁾ is to study the dynamics of ^{137}Cs in the biota of the Red Lakes, Minnesota (Lat. 48°N , Long. 95°W). The time and location at which each batch of fish was caught is well documented; thus continued sampling of these fish permits a determination of the dynamics of ^{137}Cs in the fish population. Ideally, these findings may help to establish methods for evaluating the behavior of other environmental pollutants in a freshwater system.

The fish were usually received whole, and were then cleaned and prepared in bulk samples of approximately two kilograms each. Measurements for radioactivity (^{137}Cs and ^{40}K) in these samples were made by low-background gamma-ray scintillation spectrometry methods using a $4'' \times 4''$ NaI (Tl) crystal and a 400-channel analyzer.

PROGRESS REPORT

During the summer months, the Red Lakes Fisheries Association catches and markets three varieties of fish: perch, pickerel, and northern pike. For the past three summers, samples of these fish have been analyzed for their ^{137}Cs and potassium content. Table 105 shows the results of these measurements for the summers of 1966 and 1967. Table 106 gives average values for the years 1965 through 1967.

These data show nearly constant levels of ^{137}Cs in pickerel and northern pike during 1966 and 1967, and in perch from the 1965 through 1967 seasons. The specimens labeled northern pike in 1965 were filleted, and are suspect in that they may have actually been pickerel. The relatively constant levels of ^{137}Cs in fish from the Red Lakes indicates, in turn, the existence of a nearly constant pool of biologically available ^{137}Cs in the lake. The direct input of ^{137}Cs from new fallout has decreased by an order of magnitude from 1965 to 1967. However, the total accumulation of this radionuclide in this lake system has remained essentially unchanged during this time—small losses via the outflow being matched by the new inputs. Thus it seems reasonable to relate the biologically available

TABLE 105. ^{137}Cs AND POTASSIUM CONCENTRATIONS OF FISH FROM THE RED LAKES, MINNESOTA

Type of fish	Date caught	^{137}Cs , pCi/kg	Potassium, g/kg	^{137}Cs /potassium, pCi/g
Perch	7-31-66	1175 ± 11	2.88 ± 0.07	408
Northern pike	"	2088 ± 12	3.07 ± 0.06	680
Pickerel	"	2760 ± 16	3.24 ± 0.08	852
Perch	8-13-66	1114 ± 10	2.87 ± 0.07	388
Northern pike	"	1542 ± 11	3.21 ± 0.07	480
Pickerel	"	2265 ± 15	3.18 ± 0.07	712
Perch	8-26-66	1680 ± 12	3.25 ± 0.07	517
Northern pike	"	1233 ± 9	3.31 ± 0.06	373
Pickerel	"	2261 ± 14	3.20 ± 0.07	707
Perch	6-17-67	1701 ± 13	3.00 ± 0.07	567
Northern pike	"	1567 ± 11	3.27 ± 0.07	479
Pickerel	"	2593 ± 14	3.04 ± 0.07	853
Perch	6-30-67	1644 ± 13	3.21 ± 0.08	512
Northern pike	"	1775 ± 11	3.29 ± 0.07	540
Pickerel	"	2580 ± 15	3.10 ± 0.08	800
Perch	7-14-67	1008 ± 9	3.04 ± 0.07	332
Northern pike	"	1508 ± 10	3.19 ± 0.06	473
Pickerel	"	2529 ± 14	3.32 ± 0.07	762
Perch	8-25-67	1484 ± 11	3.09 ± 0.07	480
Northern pike	"	1750 ± 11	3.53 ± 0.07	496
Pickerel	"	2326 ± 13	3.10 ± 0.06	750
Perch	7-29-67	1012 ± 9	3.18 ± 0.07	318
Northern pike	"	1041 ± 9	3.43 ± 0.07	303
Pickerel	"	2325 ± 13	3.37 ± 0.07	690

pool of ^{137}Cs to some fraction of the total accumulation.

The dependence of the ^{137}Cs content of fish in a freshwater system upon the ^{137}Cs accumulation in that system is in direct contradiction to the general behavior of ^{137}Cs observed in terrestrial systems. ^{137}Cs is readily absorbed by clay particles in soil, and is thus not available for plant uptake. The ^{137}Cs which is present in plant and animal tissues comes almost entirely from the direct deposition (i.e., fallout) on plant surfaces, with subsequent ingestion by animals.

TABLE 106. AVERAGE ^{137}Cs AND POTASSIUM CONCENTRATIONS OF FISH FROM THE RED LAKES, MINNESOTA

	Summer 1965			Summer 1966			Summer 1967		
	^{137}Cs , pCi/ kg	K, g/kg	$^{137}\text{Cs}/\text{K}$, pCi/g	^{137}Cs , pCi/ kg	K, g/kg	$^{137}\text{Cs}/\text{K}$, pCi/g	^{137}Cs , pCi/ kg	K, g/kg	$^{137}\text{Cs}/\text{K}$, pCi/g
Perch	1149	2.94	391	1323	3.00	441	1370	3.10	442
Northern pike	3620 ^(a)	3.33	1087	1621	3.20	507	1528	3.34	457
Pickrel	—	—	—	2429	3.21	757	2450	3.19	768

^(a) Because these fish were obtained as filets they were not identified as accurately as whole fish specimens; hence they could have been pickerel (walleyed pike).

The strong dependence of the ^{137}Cs content of terrestrial biota upon fallout rate rather than total accumulation is illustrated by the changes in the levels of ^{137}Cs in the human diet. The adult diet in Chicago

area, which contains predominantly soil-dependent products, has decreased from an average intake of 146 pCi ^{137}Cs /day in 1965 to less than 40 pCi/day in 1967.^(2, 3)

The expectation is that the current downward trend of ^{137}Cs in the human diet will continue (with an effective half-time of ~ 18 months), whereas the ^{137}Cs level in the Red Lakes fish will decrease at a much slower rate over the next few years.

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MEASUREMENT OF OZONE IN SURFACE AIR AT ARGONNE NATIONAL LABORATORY

Surmukh S. Brar, Donald M. Nelson, and Philip F. Gustafson

PURPOSE AND METHODS

Ozone is produced in the upper atmosphere by the action of ultraviolet light on oxygen, and at ground level it is formed by the action of sunlight on industrial and automobile exhausts.⁽¹⁾ It is found in relatively large concentrations (at times 1 ppm in air) in smog⁽²⁾ areas, such as Los Angeles, California, and is an indication of local atmospheric pollution. Ozone is highly toxic to man (threshold limit value 0.1 ppm in air⁽³⁾) and is a very strong oxidant. Because of its relatively discrete sources of production (i.e., urban industrial areas and the upper atmosphere) ozone has potential use in tracing of air movements in a manner analogous to that of radioactive fallout. For these reasons, ozone studies in ground level air were initiated as part of the Environmental Studies Program at Argonne, Illinois.

PROGRESS REPORT

A recording, ozone-sensing meter* detects ozone by oxidation of potassium iodide.⁽⁴⁾ This meter is simple to operate and requires attention twice a week. The unit is installed in a second-floor laboratory near

* Model H-100-LC Ozone Meter purchased from the Welsbach Corporation, Philadelphia, Pennsylvania 19129.

FIG. 220.—Average daily concentration of ozone in surface air at Argonne, Illinois.

a north window. Air for the detection of this gas is drawn from the outside into the sensing element through Teflon tubing. The intake is located far enough out in the air that decomposition effects on ozone by obstructions present in the vicinity are minimized.

Figure 220 and Table 107 give the daily average values of ozone at Argonne in parts per billion against time in days from mid-May to mid-September 1967. The data are too limited to allow a correlation with meteorological variables or to identify any seasonal

TABLE 107. AVERAGE DAILY CONCENTRATION OF OZONE IN SURFACE AIR AT ARGONNE IN PARTS PER BILLION

	Sun-day	Mon-day	Tues-day	Wednes-day	Thurs-day	Friday	Satur-day
May				10.1	19.6	19.8	15.4
	3.8	5.4	3.9	10.4	11.0	12.4	25.1
	21.8	—	—	—	—	—	—
June	—	—	—	14.7	18.1	14.2	10.5
	8.2	17.3	25.0	11.2	21.0	19.8	6.2
	16.9	7.0	—	—	—	20.8	18.9
July	17.8	16.0	14.5	11.8	27.2	15.6	13.8
	20.7	25.6	20.0	15.6	—	—	16.7
	10.3	23.9	11.2	10.9	4.1	16.6	17.6
	13.7	25.0	18.3	13.6	26.4	11.8	19.4
	18.7	15.9	27.8	23.9	17.3	16.8	16.6
	17.7	20.7	15.1	20.1	11.0	14.7	20.0
	27.2	25.6	19.3	17.9	14.8	9.0	4.6
Aug.	17.3	13.2	6.7	16.4	14.1	20.3	19.2
	11.7	15.1	9.7	7.2	12.8	8.8	19.5
	19.6	21.0	21.4	25.2	14.0	17.9	14.9
Sept.	9.2	—	28.2	20.8	16.4	21.3	14.4
	20.0	23.2	4.9	7.1	13.9		
Average	15.9	18.2	16.1	14.8	16.1	16.0	15.8

FIG. 221.—Ozone concentration plot for August 20, 1967 in surface air at Argonne, Illinois.

variations at this time. Table 107 was prepared to ascertain whether ozone production varied with specific days in the week (i.e., weekdays relative to weekends). The table does not reveal significant trends. This observation may be interpreted to mean that either the locally produced ozone is small, in relation to the total present, or to suggest that high, as well as low concentrations of local ozone are produced equally on weekends and on weekdays, when the laboratory is operating at full capacity. Typically, ozone concentration in air begins to rise early in the morning, builds up to a maximum past mid-day, and falls to a low level by evening, where it remains through the night. Figure 221 shows a plot of an average diurnal cycle (August 20, 1967).

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