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RADIATION MORTALITY IN THE MOUSE: KINETICS OF
INJURY ACCUMULATION DURING
PROTRACTED EXPOSURES
IN THE SUPRALETHAL RANGE

S. Phyllis Stearner and Margaret H. Sanderson

Radiation death after γ -ray exposures in the 30-day lethal range occurs almost exclusively between 9 and 30 days;⁽¹⁾ it is associated with functional failure of the hematopoietic system and apparently involves principally anemia and bacteremia.^(2,3) With increase in radiation dose (in the so-called 30-day supralethal range), survival time decreases to a mean value of about 4.5 days and then remains constant for additional doses as great as several thousand roentgens; damage to the intestinal epithelium is the characteristic symptom.⁽⁴⁻⁶⁾ There is evidence that the immediate cause of death is associated with excessive loss of water and electrolytes and resultant fluid imbalance.⁽⁷⁻¹²⁾ In most instances bacterial infection appears to be a minor factor contributing to death within this period.^(2,12,13) Exposure to doses between the LD₅₀ for the 9-30 day lethality and the LD₅₀ for death within 4-6 days commonly results in a mean survival time of 7 or 8 days, with severe bacteremia a common finding.^(6,13,14)

In a previous report an empirical model was proposed that described the accumulation of lethal injury during radiation, for doses in the 30-day lethal range.⁽¹⁾ Two processes were postulated, the first dependent on exposure time (reversible) and embodying a constant, linear reversal of radiation effects, and the second independent of exposure time for periods through at least 24 hours (irreversible). Mathematical description of the time-dependent process is given by

$$\frac{D}{D_0} = 1 + \frac{t}{\tau}, \quad (1)$$

where D/D_0 is the ratio of a dose (D) protracted over a time period t to the instantaneously delivered dose (D_0) that produces the same mortality; τ is a species parameter that relates radiosensitivity and reversal rate.

The studies reported here are concerned with a kinetic description of radiation injury accumulation associated with lethality expressed within 9 days after exposure. The descriptive analysis is limited to single protracted doses of Co⁶⁰ γ -rays and is based on the same model that was used for 9-30 day lethality.⁽¹⁾

Methods

Six-week-old LAF₁ mice, male and female, were obtained from the Jackson Memorial Laboratory, Bar Harbor, Maine. At 120 ± 7 days they were exposed to γ -rays from one of three Co⁶⁰ sources: 5000-, 1000-, or 100-curie. Conditions of irradiation and experimental procedures were as previously described.⁽¹⁾ The results reported here are based on single doses of 900 to 3200 r, protracted over 9 different exposure periods ranging from 60 to 1440 min. There were 93 dose-exposure-time groups; most contained 60-72 animals, with equal numbers of each sex. A total of approximately 5500 irradiated animals is represented in the analysis. Time of death was computed from the beginning of the exposure. Gross necropsies were performed on randomly selected animals from each dose-time group. In some instances, segments of duodenum and ileum were examined microscopically.

In groups given antibiotic therapy, each mouse received 5 mg streptomycin in 0.25 ml saline subcutaneously once each day, beginning on the first day after irradiation. Corresponding controls received an equivalent volume of saline or nothing. For determination of the daily incidence of bacteremia, 200 animals from a separate sacrifice series were used. Abdomen and chest were opened aseptically and blood was aspirated from the heart with a sterile syringe. Some blood was streaked on the surface of a blood agar plate and the remaining blood was inoculated into nutrient or brain-heart infusion broth. Cultures were incubated for 24 hr; then all broth tubes were streaked onto EMB agar. Individual colonies were isolated from both types of plates and were inoculated into various special broth media for identification of organisms. Negative plates were incubated for an additional 24 hr. No anaerobic cultures were made since it has been reported that very few anaerobes are found in blood cultures.⁽¹⁴⁾

Characteristics of the Principal Symptom Complexes

The pattern of daily mortality after single exposures over a wide dose range reveals a clustering of deaths around certain characteristic survival times. When the mean survival time is about 5 days, the mortality is concentrated within 4-6 days after exposure; after doses in the 30-day lethal range, most of the mortality occurs within the period 9-16 days. Between these extremes, in groups exposed to intermediate doses, there is a clustering of deaths between 6 and 9 days. Mortality in the 4 to 6-day period, described as "acute intestinal death," is characterized by extensive loss of intestinal epithelium. The intermediate period (6-9 days) is not always recognized as a distinct entity, but appears to be neither a late intestinal nor an early marrow death.

After doses of Co^{60} γ -rays in the 4- to 6-day lethal range, severe denudation of intestinal epithelium is found in decedents at 4 days. By the 6th day evidence of extensive repair is frequently seen, or even fairly normal intact mucosa may be found. That there had been previous damage to the intestinal mucosa is indicated by the abnormal architecture. Although large segments of denuded intestinal epithelium can be tolerated for long periods, (6) most evidence indicates that severe fluid and electrolyte loss frequently occurs in the 4- to 6-day period and is important in the critical lethal event. (2, 7-11)

Despite evidence of extensive repair of the intestinal mucosa on the 6th day, a high incidence of bacteremia was observed in animals that survived to the 6th or 7th day. In order to assess the role of infection in the mortality occurring in the first 9 days after exposure, the effect of protective treatment with streptomycin was tested at radiation levels at which 4- to 6-day mortality predominates and at levels at which 6- to 9-day mortality predominates. In addition, the incidence of positive blood cultures in these groups was determined for comparison with mortality studies. After a 60-min exposure at a dose level within the range for intestinal death, the daily incidence of bacteremia within the first 4 days did not exceed 20%, and streptomycin treatment resulted in almost no change in mean survival time (from 5.7 to 5.9 days). After a somewhat lower dose, where 6- to 9-day mortality predominated, the principal mortality occurred within 9-13 days when streptomycin therapy was given. Daily mortality in treated and untreated irradiated groups is shown in Table 1. In untreated irradiated groups, almost all animals tested had positive blood cultures by day 7. Bacteria were usually recovered in pure culture and were principally gram-negative rods commonly found in the intestinal tract. In streptomycin-treated animals, the incidence of bacteremia was less than 25%, and the organisms represented were shown to be drug-resistant.

TABLE 1

Effect of streptomycin therapy on daily mortality
after irradiation

Streptomycin therapy*	Mortality, %												Mean survival time (days)
	Days after irradiation												
	N	4	5	6	7	8	9	10	11	12	13		
2800 r**													
0	70		37.1	58.6	4.3								5.7
+	40	5.0	22.5	55.0	10.0	7.5							5.9
1900 r**													
0	108	5.5	3.7	11.1	25.0	26.8	22.2	5.5					7.5
+	54		3.7	7.4	3.7	3.7	7.4	18.5	27.8	22.2	5.5		10.1

*Dose: 5 mg streptomycin in 0.25 ml saline solution per mouse, administered daily beginning on day 1

**60-min exposure

Analysis

Lethality in the 4- to 6-day period

Based on the correlation between daily distribution of deaths and characteristic pathology, the sixth day after irradiation appears to represent the maximum survival time for expression of the intestinal mode of death and the minimum time for a later radiation death associated with intermediate doses. The total mortality occurring on day 6 is, then, the result of these two lethal mechanisms. To minimize the error resulting from overlap in time of death, the mortality on the sixth day has been partitioned on the basis of the relative magnitudes of the mortality on the fifth and seventh days. There is, of course, a difference in the amount of mortality in this period depending on whether or not all 6-day deaths are included. It can be demonstrated, however, that the kinetic relations of dose and exposure time for injury accumulation are unaltered when analysis is based on the entire 4- to 6-day mortality. Incidentally, no deaths occurred before the fourth day in the entire dose-exposure-time series reported here.

Females showed a consistently greater sensitivity than males; the difference was dependent on exposure time. The average shift in LD_{50} was 100 r. Constants of the exposure-time effect, however, were not significantly different. In the statistical analysis, mortality data for the males have been adjusted for the sex difference in sensitivity, and computations are based on combined data.

The postulates upon which the following analysis is based have been previously stated for 9- to 30-day lethality. (1) Dose-mortality relations for all exposure periods are shown as probit transforms in Figure 1. In groups exposed for 360 min or less, lethality is assumed to

Figure 1

Probit transform of 4- to 6-day mortality (%), versus dose, by exposure-time group. Dashed line, estimated mortality distribution for instantaneously delivered doses ($LD_{50} = 1642$ r, $\sigma = 228$ r). Solid lines, theoretical curves. See explanation in text.

express time-dependent injury only. The effect of time-independent lethal processes is seen in groups exposed over longer periods, and this effect predominates for protractions of 960 and 1440 min. The effect of exposure time on dose-mortality relations for exposure periods through 360 min is used to describe the kinetics of injury accumulation for the time-dependent effect. The distribution of mortality with respect to dose in groups exposed for 60 min is used as the reference point in the empirical determination of the D'/D'_{60} ratios.* The mean ratios for exposure times through 360 min are given in Table 2. The relation between mean D'/D'_{60} ratios and exposure time (Figure 2) shows a constant linear reduction of

TABLE 2

Mean D'/D'_{60} ratios for mortality within
4 to 6 days after irradiation

Exposure time, min	Number of groups	Mean ratio	Standard deviation
60	15	1.0080	0.04381
90	13	1.0546	0.06073
120	12	1.0916	0.06309
180	9	1.1349	0.05886
240	10	1.2915	0.08092
360	10	1.3529	0.07107

Figure 2. Relation between mean D'/D'_{60} and exposure time.

The slope of the least-squares line, forced through

1.00 at $t = 60$ min, is 0.0012854 ± 0.0000761 .

$1/b = 778$ min, $\hat{\sigma} \ 1/b = 61$ min.

*The notations prime and double prime are used to distinguish variables of the 4- to 6- and 6- to 9-day periods from each other and also from corresponding variables of the 9-30 day period previously described.(1)

dose-effectiveness with increase in exposure time. The curve was fitted by the method of least squares, with the restriction that $D'/D'_{60} = 1.000$ at $t = 60$ min. From the slope of this curve, defined as $1/(\tau' + 60)$ (where τ' is the time constant), the estimate of τ' is determined to be 718 ± 61 min. The estimated D'/D'_0 line (Figure 2) has a slope equal to the reciprocal of τ' . The estimated mortality distribution for instantaneously delivered doses* shown in Figure 1 has $LD_{50} = 1642$ r and $\hat{\sigma} = 228$ r. The curves in Figure 1, other than that for the 60 min groups, are theoretical and are based on constants of the least-squares line of Figure 2 corresponding to each exposure time. From the postulate of a linear reversal rate, the rate of dose reduction is constant per unit time during irradiation and independent of amount of accumulated dose. The mean of the normally distributed reversal rates is 2.29 r/min; that is, 50% of the animals can reverse the effects with which mortality in this period is associated at a rate equal to or greater than 2.29 r/min.

To obtain an estimate of sensitivity to time-independent injury mechanisms alone, the assumption of independence of effects was applied. In groups exposed for 480, 960 and 1440 min, the contribution of the reversible effect was removed from the total mortality. Computations were based on the formula

$$R_{d,t} = P_{d,t} + Q_d - P_{d,t}Q_d, \quad \text{or} \quad Q_d = \frac{R_{d,t} - P_{d,t}}{1 - P_{d,t}} \quad (2)$$

where $R_{d,t}$ is the total proportion of animals dying from a dose d protracted over time t , and $P_{d,t}$ and Q_d are the proportion dying of time-dependent and time-independent effects, respectively.⁽¹⁾ The resultant mortality, assumed to represent expression of the time-independent lethal injury, is approximately normally distributed with respect to dose (Figure 3) and is characterized by the least-squares line fitted to the probit transform for the combined groups. Least-squares constants are $LD_{50} = 2838 \pm 35$ r and $\hat{\sigma} = 394$ r.

Figure 3

Probit transform of percent mortality, versus dose, for the time-independent (irreversible) effect expressed in the 4- to 6-day period. Constants of the least-squares line for the combined are $LD_{50} = 2838 \pm 35$ r; slope = 0.0025395 ± 0.0002991 ; $\hat{\sigma} = 394$ r.

*Calculated from the relation $D_0 = \tau' / (\tau' + 60)(D_{60})$. (See Reference 1.)

Lethality in the 6- to 9-day period

The mortality pattern in the 6- to 9-day period is less stable than that in the 4- to 6- or the 9- to 30-day periods and probably reflects contamination from lethal mechanisms that are predominantly expressed in the earlier and later periods. For this reason determinations of the constants are subject to greater error. In spite of this, however, the general description of the dose-exposure-time relations reveals a pattern similar to that observed for the other mortality periods. Since there was no apparent difference in response between males and females in this period, data from the two sexes have been combined.

Deaths in this period predominated after radiation doses that were intermediate in the range covered in this study. Dose-mortality relations (probit transforms) for all exposure times are shown in Figure 4. The presence of time-dependent and time-independent lethal processes are indicated as in the earlier period, but the time effect was of somewhat smaller magnitude. Again, the dose-mortality distribution for groups exposed for 60 min was used as the basic exposure time series. Table 3 gives the mean D''/D''_{60} ratios for exposure times through 360 min, and Figure 5 the relation between mean D''/D''_{60} ratios and exposure time. From the slope of the least-squares line the estimate of τ'' was calculated to be 963 ± 104 min. The estimated D''/D''_0 line in Figure 5 has a slope equal to the reciprocal of τ'' . The estimated mortality distribution for instantaneously delivered doses shown in Figure 4 has $LD_{50} = 1385$ r and $\hat{\sigma} = 178$. All curves, other than that for the 60-min groups, are theoretical, based on D''/D''_{60} values in Figure 5 that correspond to each exposure time. For 6- to 9-day mortality the mean of the normally distributed reversal rates is 1.44 r/min.

Figure 4. Probit transform of the 6- to 9-day mortality (%) versus dose, by exposure-time group. Dashed line, estimated mortality distribution for instantaneously delivered doses ($LD_{50} = 1385$ r, $\hat{\sigma} = 178$ r). Solid line, theoretical curves. See explanation in text.

TABLE 3

Mean D''/D''_{60} ratios for mortality within
6 to 9 days after irradiation

Exposure time, min	Number of groups	Mean ratio	Standard deviation
60	9	0.9858	0.062347
90	5	1.0776	0.066890
120	4	1.0655	0.050948
180	8	1.1549	0.057613
240	9	1.1700	0.045604
360	9	1.2814	0.074572

Figure 5. Relation between mean D''/D''_{60} and exposure time.

The slope of the least-squared line, forced through

1.00 at $t = 60$ min is 0.00097749 ± 0.0000994 .

$1/b = 1023$ min, $\hat{\Delta} \ 1/b = 104$ min.

To estimate the sensitivity to time-independent injury mechanisms expressed in this period, the contribution of the reversible effect was removed from the total mortality in groups exposed over 480, 960 and 1440 min. Computational procedure was the same as for the 4- to 6-day period. The dose-mortality relation for the resultant time-independent lethal injury (Figure 6) appears to be normally distributed, and is characterized by the least-squares line fitted to the probit transform for the combined groups. Constants are $LD_{50} = 2369 \pm 35$ r, and $\hat{\Delta} = 462$ r.

Figure 6. Probit transform of per cent mortality versus dose for the time-independent (irreversible) effect expressed in the 6- to 9-day period. Constants of the least-squares line for the combined groups are $LD_{50} = 2369 \pm 35$ r, slope = 0.0021660 ± 0.0001897 . $\phi = 462$ r.

Discussion

Difficulties associated with separation of the several major injury complexes that find lethal expression in the acute (30-day) postirradiation period are well recognized. Yet a kinetic description of the accumulation of effect for each of the principal modes of injury is of value in characterizing the different injury processes. It is hard to overcome problems such as distinguishing the various modes of acute death and estimating the extent of possible interactions in the actual production of lethality. That differences in sensitivity and kinetics do exist is clearly evident, and they have been described.^(2,15,16) Nevertheless, adequate indicators are not available for identifying the predominant injury or injuries in each individual animal that succumbs to radiation effects, and indirect methods of separation are required. Errors in the mortality values for a specific mode of death, when partition is based on time alone, arise from random shifts in the modal time of death. In general, however, this approach yields a stable response pattern, especially for the 4- to 6-day period. It appears to involve a minimal amount of error and to provide a procedure for separating at least the major component processes expressed in the acute mortality.

The time-dose pattern of mortality, taken together with the times of occurrence of intestinal lesions and bacteremia, supports the thesis that the 4- to 6-day mortality is largely distinct from that which occurs later. The severely damaged intestinal mucosa, with which mortality in

the 4- to 6-day period is associated, is frequently repaired after 6 days. This is found to be the case even in decedents. It would appear that breakdown of the intestinal barrier due to loss of epithelium is of little importance in the initiation of bacteremia that develops after several days. Small numbers of enteric organisms normally penetrate the intestinal lining but are unable to multiply in the circulating blood.⁽²⁾

Early failure of normal body defense mechanisms may be the critical factor in development of the infection. In most cases only a single species of microorganism was isolated. Thus, it appears that multiplication must occur in the blood stream, resulting in the overwhelming bacteremia. In dose groups in which mortality was concentrated in the 6- to 9-day period, streptomycin therapy greatly reduced mortality in this period. Yet there were no survivors beyond 14 days (unpublished results). Hematopoietic failure, probably chiefly anemia, may account for this mortality in the absence of infection. Similar results were reported by Miller, et al. who found a high incidence of bacteremia after 6-7 days when the radiation dose was sufficient to produce extensive mortality on days 8-9; streptomycin therapy was found to be most effective in reducing this mortality.^(14,17) After a lower radiation dose (slightly more than the $LD_{50/30}$), the highest incidence of bacteremia occurred after 10-11 days and was never as severe as that observed after larger doses. Drug therapy was of more limited value in reducing mortality in this period; some deaths always occurred in spite of treatment.

Our kinetic model of injury accumulation during irradiation has been used previously in a statistical description of the exposure time effect for 9- to 30-day mortality.⁽¹⁾ Extension of the model to other mortality periods permits comparison of reversal kinetics for different lethal complexes. A rapid reversal or repair of the radiation effect is characteristic of intestinal injury. This is indicated by a value of 719 min for the reversal constant τ' , compared with 1419 min for the injury associated with 9- to 30-day mortality;⁽¹⁾ for the intermediate 6- to 9-day mortality, τ'' is 963 min.

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THROMBI IN THE LEFT ATRIUM

R. J. Michael Fry, Katherine Hamilton, and Hermann Lisco

In a previous report⁽¹⁾ a description was given of a cardiac lesion found in inactive BALB/c female breeders. The lesion consists of one or more thrombi, in various stages of organization, in the left atrium. A number of thrombotic episodes may occur with a considerable time lapse between episodes. There appears to be a correlation between the number of litters and the number of thrombi. For this reason and others it is believed that the thrombus formation is associated with pregnancy or parturition. These findings have been confirmed in another colony.⁽²⁾ The incidence of this lesion, approximately 60%, in retired female breeders, is similar in the two colonies. We have also found the same type of lesion in virgin female and in male BALB/c mice, however. The following breeding experiment was carried out to ascertain whether a genetic factor was involved. A BALB/c male was crossed with a C57BL/6 female and the F₁ progeny were bred with the BALB/c mice. The progeny of each successive generation were similarly bred with mice of the BALB/c parent strain. Six backcross generations were produced, each with an increased percentage of the BALB/c strain. The results, shown in Table 4, indicate that with an increasing percentage of BALB/c in the female mice the probability of thrombus formation increases. This is only known to be true if the female mice resulting from the backcross breeding are themselves used for breeding. It is not known whether the genetic make-up of the hybrid influences the incidence of this lesion in the virgin female or

TABLE 4

Incidence of thrombus in successive backcross generations

Generation	% BALB/c	No. females	No. with thrombi	%	No. males	No. with thrombi
1	75.0	3	0	0	2	0
2	87.5	9	2	22	7	0
3	93.8	6	2	33	5	0
4	96.9	7	3	43	6	0
5	98.4	10	6	60	6	0
6	99.2	17	13	76	18	1

male mice. Furthermore the association between the genetic factor and the pathogenesis of thrombus formation is not known. The genetic factor may be related to coagulation and the changes that occur in this during pregnancy and parturition. On the other hand, the architecture of the left atrium may be affected.

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THE INTESTINAL FLORA OF THE MOUSE

R. J. Michael Fry, Patricia A. Brennan, and Thomas E. Fritz

It is now desirable, if not essential, to know the spectrum and distribution of the intestinal flora of animals that will be used for irradiation and intestinal cell general experiments.

There is considerable variation in the number and types of bacteria in mice maintained under different environments. The present investigation has been concerned with the following:

- 1) Establishing the types, distribution, and numbers (estimates) of bacteria in the various regions of the small intestine of CF #1 and BCF₁ mice;
- 2) Determining the influence of various factors, such as starvation or antibiotics, on the distribution of the flora.

The results so far confirm the relationship between number and distribution of bacteria and intestinal motility. The upper small intestine, although not sterile, has a smaller bacterial population than the more distal regions. This is due, in part, to the flushing effect of the rapid passage of intestinal contents. The number and distribution of bacteria is markedly altered in mice maintained on glucose or water for 24 hours prior to investigation. In a previous investigation by one of us, it was found that neomycin was effective in eliminating the majority of bacteria from the colon and lower small intestine but not from the upper part of the small intestine. Our preliminary findings appear to confirm this finding.

POPULATION KINETICS OF NEOPLASTIC MAST CELLS (P815Y) AFTER X-IRRADIATION

G. Mark Kollmorgen

Neoplastic mast cells (P815Y) have been grown both in vivo (peritoneal cavity of DBA/2 mice) and in vitro after exposure to X-irradiation in an attempt to determine the dependence of killing and mitotic lag on dose. X-ray doses of 62.5, 125, 250, 500, 750 and 1000 r were given.

Control mastocyte cultures have a doubling time of about 15 hours, reproduce as free-living cells without attachment to glass surfaces, and fully retain their strain-specific tumor-inducing properties and characteristic biochemical functions after extended periods of reproduction in culture. After an LD₅₀ dose (about 130 r), cells in culture return to normal growth rate in approximately 45 hours. With larger doses more time is required. When cells are irradiated and grown in vivo, the size of the inoculum required to cause death of the host is increased as a function of X-ray dose. Furthermore, the higher the X-ray dose, the longer the survival time of the host animal.

The observed patterns of survival and regrowth are being analyzed in terms of a statistical model that incorporates parameters for generation time and sensitivity of stages in the generation cycle.

EFFECTS OF HIBERNATION ON THE LATENT PERIOD IN NORMAL AND X-IRRADIATED GROUND SQUIRRELS

Bernard N. Jaroslow and Douglas E. Smith

In experiments designed to examine the details of the immune responses in hibernators, we have studied the disappearance of I^{131} -labeled bovine serum albumin (BSA) from the circulation of ground squirrels (Citellus tridecemlineatus).

All animals received a single intraperitoneal injection of 10 mg of I^{131} trace-labeled BSA or 1 ml of I^{131} trace-labeled normal ground-squirrel serum (method of Talmage et al.).⁽¹⁾ The radioactivity of the BSA solution was 99% precipitable by 5% trichloroacetic acid.

Blood samples from the tail were collected on tared filter paper, air dried overnight, weighed, and counted in a well-type scintillation counter. After the counts were corrected for background, decay, and weight of sample they were converted to per cent of activity of the first sample taken after antigen injection and plotted against time on a semilogarithmic scale. The antigen disappearance rate (ADR) is calculated from the slope of the line. As an indicator of the relative ADR, all of our data are presented in terms of the half-disappearance time ($t_{1/2}$) in days. In ground squirrels, antigen disappearance occurs in three distinct phases marked by sharp changes in ADR. The phases are similar to those observed in rabbits by Dixon et al.,⁽²⁾ who termed them 1) the equilibration phase, 2) the nonimmune disappearance phase, and 3) the immune disappearance phase - marked by an accelerated ADR that signifies the end of the latent period and the appearance of antibody. Phase 1 was not usually observed in the present studies because the first sample was taken 1 or 2 days after antigen injection.

Both hibernating and control squirrels were irradiated in chambers maintained at 5°C. They were given a single total-body X-ray dose of 450 r (250 KV, 15 ma; 0.5 mm Cu + 3 mm bakelite filters; 1.7 mm Cu half-value layer; 35 cm target distance; 128 r/min).

Results

In 14 ground squirrels maintained in quarters at 23°C, the mean $t_{1/2}$ of trace-labeled BSA during the nonimmune phase was 9.4 ± 2.7 days. At the end of the latent period, which had a mean value of 7.1 ± 0.3 days, the immune disappearance phase began and the mean $t_{1/2}$ decreased to 1.8 ± 0.2 days.

Ground squirrels were injected with BSA and immediately put in the cold room for 14, 28, or 56 days. The ADR during this period was highly variable and was probably related to the amount of time spent in hibernation. In this and succeeding experiments, we used data only from those animals in which the ADR was very slow ($t_{1/2} > 87$ days) while in hibernation, regardless of the number of observed spontaneous arousals.

The data in Table 5 indicate that the ADR is similar for all experimental groups during each phase and that there were no significant differences between groups ($P > 0.05$). In animals that hibernated for 14 days, the mean posthibernation latent period was significantly shorter than the latent period in controls (Table 5), $P < 0.001$. After hibernation, for 28 or 56 days the posthibernation latent period ranged from 0-9 days.

TABLE 5

Antigen disappearance (I^{131} -BSA) and the posthibernation latent period in ground squirrels after arousal from hibernation*

Treatment group**	No. of animals	Nonimmune, $t_{1/2}$ days $\dagger, \dagger\dagger$	Immune, $t_{1/2}$ days $\dagger$	Posthibernation latent period, $\dagger, \dagger\dagger$ days
1. No hibernation	14	9.4 ± 2.7	1.8 ± 0.2	7.1 ± 0.3
2. 14-day hibernation	10	4.1 ± 0.3 (10)	2.1 ± 0.2	5.7 ± 0.2
3. 28-day hibernation	5	4.4 ± 1.2 (4)	1.8 ± 0.3	7.3 ± 0.8 (4)
4. 56-day hibernation	8	3.2 ± 0.3 (4)	1.6 ± 0.7	5.8 ± 1.1 (4)
5. Hibernation 4 days after injection	8	3.7 ± 0.2 (6)	1.7 ± 0.1	8.0 ± 0.4 (6)
6. Hibernation 6 days after injection	6	2.9 ± 0.2 (2)	1.2 ± 0.2	5.0 ± 0 (2)

* Only those animals were used in which the $t_{1/2}$ of antigen was longer than 87 days during hibernation.

** Animals in Groups 2-4 were placed in the cold room immediately after antigen injection, those in Groups 5 and 6 were placed in the cold room for 18 and 14 days, 4 and 6 days after antigen injection, respectively.

$\dagger$ The figures represent the Mean $\pm$ S. E.

$\dagger\dagger$ Numbers in parentheses indicate the number of animals that had a measurable posthibernation latent period. The remainder, upon arousal, eliminated antigen at the immune rate.

Ground squirrels were placed in the cold room 4 and 6 days after antigen injection for 18 and 14 days, respectively. In the former group, the posthibernation latent period was 0 days in 2 animals and essentially normal (6.5 to 9.3 days) in 6 animals; in the latter group, it was 0 days in 4 animals and 5 days in 2 animals. It was, therefore, apparent that the events of the latent period that had occurred before entry into hibernation were completely or partially abrogated during hibernation in 75% of one group and in 33% of the other (see Table 5).

Since X-irradiation at different times before or after antigen injection may suppress or increase antibody-forming capacity,⁽³⁾ and radiation injury may develop during hibernation,⁽⁴⁾ we investigated the effect of hibernation on radiation-induced injury to the immune processes. Four groups of hibernating squirrels were treated in the following manner: Group A, 4 animals were injected with BSA, allowed to continue in hibernation for 14 days, irradiated with 450 r, and then placed in a room at 23°C; Group B, 7 animals were injected with BSA, placed in a room at 23°C, irradiated with 450 r one day later and returned to a room at 23°C; Group C, 8 animals were irradiated with 450 r, placed in a room at 23°C and injected with BSA one day later; Group D, 8 animals were irradiated with 450 r, allowed to continue in hibernation for 14 days, and then injected with BSA and placed in a room at 23°C.

All four groups showed uniform inhibition of antibody production.

Only 4 of 27 animals (Figure 7), 1 in Group A, 2 in Group B, and 1 in Group C gave evidence of a phase of immune disappearance. In all 4, the latent period was longer than normal (12.4, 10.6, 13, and 10 days, respectively). Examples of anergic animals are shown in Figure 7. The mean non-immune $t_{1/2}$ in each of the groups was A, 3.6 ± 0.5 ; B, 3.5 ± 0.4 ; C, 3.7 ± 0.6 ; and D, 3.3 ± 0.1 days. A curious phenomenon was observed in the animals of Group A. There was 50-70% decrease of circulating antigen during the first 2 days after irradiation and then the mean $t_{1/2}$ rose to values typical for all groups (Figure 7).

To determine whether the observed ADR for the first few days after arousal from hibernation was comparable to that before hibernation, we injected 9 ground squirrels with I^{131} -labeled normal ground-squirrel serum. Five squirrels were placed in hibernation for 14 days on the fourth day after injection and the remaining four squirrels were maintained at 23°C. Values of the mean $t_{1/2}$ of the I^{131} -labeled normal serum before (3.9 ± 0.6 days) and after (3.5 ± 0.3 days) hibernation and in the nonhibernating group (3.3 ± 0.2 days) were not significantly different.

Figure 7. Antigen disappearance in ground squirrels irradiated with 450 r, 14 days or 1 day, before or after antigen injection. Letters refer to the group. Numbers indicate the $t_{1/2}$ for each phase of antigen disappearance. - - - during hibernation in room at 5°C; — after arousal from hibernation in room at 23°C

Discussion

Antigen disappearance is much slower in hibernating than in non-hibernating ground squirrels. That the events of the latent period can take place during hibernation is shown by the lack of a posthibernation latent period in some of the animals that hibernated for 56 days.

The normal posthibernation latent period observed in some of the squirrels that entered hibernation 4 or 6 days after antigen injection indicate that events of the latent period, initiated before entry into hibernation, could be completely or partially abrogated in as short a period as 18 days in hibernation and that the processes of induction recurred upon arousal.

X-irradiation with 450 r inhibited the synthesis of antibody in all groups. The pattern of antigen disappearance was the same as that observed in irradiated rabbits by Dixon et al.⁽²⁾ Those that produced antibody did so only after a delay, a response that is typical of irradiated animals.⁽³⁾ It is apparent that a 2-week hibernation period interposed between irradiation and antigen injection did not result in any detectable recovery. Irradiation after induction has occurred usually does not result in the inhibition of antibody production.⁽³⁾ In Group A, the animals were rendered anergic when irradiated 2 weeks after antigen injection. This suggests that the events of the latent period may have gone full cycle during hibernation, so that, at the time of X-irradiation, induction had not recurred. The rapid drop in circulating antigen immediately after irradiation and arousal in Group A is undoubtedly an effect of irradiation and not of arousal, as can be seen from a comparison with all the other groups, whether injected with BSA or with normal serum.

We wish to thank Miss Joan A. Stachura for her excellent technical assistance.

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PROGRESS REPORT: DISEASES AND CARE OF LABORATORY ANIMALS

I. Disease Control Program

Robert J. Flynn, Thomas E. Fritz,
Patricia C. Brennan, and Calvin M. Poole

A system of monitoring the health status of all breeding, stock, and experimental animals has been initiated.

The program of microbiologic monitoring for the presence of Salmonella sp. and Pseudomonas aeruginosa within the several rat and mouse breeding colonies is now well established. Water samples are collected from approximately 25% of the breeding cages each week and tested for P. aeruginosa; fecal samples are collected from approximately 10% of the cages each week and cultured for Salmonella sp. The incidence of P. aeruginosa infection has been very low, and the few animals that gave a positive test for this organism were recent additions from outside sources. The CF#1/An1 colony has been free of P. aeruginosa infection for many months. Similarly, the incidence of Salmonella sp. infection has been nearly zero. Hundreds of samples have been tested but only one Salmonella organism has been isolated. It was identified as S. cerro and a brief report on it follows.

The incidence of intestinal parasites remains moderate, and the elimination of these from the several colonies will depend upon the successful replacement of the breeding stock with cesarean-derived or otherwise parasite-free animals.

The control or elimination of ectoparasites from the rat and mouse breeding colonies has been another goal of the health program. To this end, all discarded breeders are now being examined for mites and lice. No lice have been detected, and only two animals have been found to be infected with mites.

Rats and mice purchased from outside vendors are similarly routinely screened for P. aeruginosa and Salmonella sp. and for internal and external parasites. The results of these tests vary; they depend on the strain and source of supply. In all cases, however, the purchased animals have a higher incidence of disease than those raised in the Division's animal facilities.

The efficiency of the washing and sanitizing operation in eliminating pathogenic microorganisms, particularly P. aeruginosa, from cages and water bottles is also being checked by daily testing of sample cages and filled bottles. The results to date indicate that no pathogenic microorganisms survive the present sanitizing procedures.

In addition, all animals that die within the breeding colonies are necropsied, and an attempt is made to ascertain the cause of death. These procedures are now being extended to include all stock animals and will be extended, whenever feasible, to include experimental animals as well.

The information that is being acquired on spontaneous diseases of the various strains will be of great value in eliminating extraneous experimental variables and will allow for better interpretation of experimental results.

II. Salmonella cerro Isolated from a Breeding Colony

Patricia C. Brennan, Thomas E. Fritz,
and Robert J. Flynn

Salmonella cerro was first isolated in Uruguay from the mesenteric lymph nodes of normal hogs⁽¹⁾ and later from infants suffering from enteritis.⁽²⁾ It has been isolated in the United States from dogs and from fowl.^(3,5)

While performing routine tests of fecal samples from the CF#1/Anl breeding colony, an organism was isolated that produced acid in mannitol and acid and gas in glucose. No change was observed in lactose or sucrose. Urea was not hydrolyzed; indole was not produced. The organism was motile, gave a positive methyl red test, utilized citrate and produced H₂S. It agglutinated with polyvalent Salmonella antiserum (Difco) but failed to agglutinate with Salmonella group specific antisera, Groups A through I. A positive ninhydrin test⁽⁶⁾ was obtained, and on that basis the culture was submitted to the Communicable Disease Center, U. S. Public Health Service, Atlanta, for serological identification. It was identified as S. cerro.

We have not been able to reisolate S. cerro from this colony. Neither has any other member of the genus been isolated subsequently from the colony.

The source of this organism and method of introduction are matters of speculation, but several possibilities exist. These include the commercial food, which is not sterilized, and contamination by the animal caretakers.

III. Studies on Pasteurella pneumotropica

Patricia C. Brennan, Thomas E. Fritz,
and Robert J. Flynn

A high incidence of conjunctivitis was observed in the CF#1/Anl mouse colony during the period covered by this report.

Routine bacteriologic screening of the affected animals resulted in the consistent isolation of a gram-negative organism that produced acid but no gas in glucose, lactose, sucrose and maltose broths, and that did not ferment mannitol. It did not grow on eosin methylene blue agar, and it was indol and urease positive and methyl red negative. On the basis of these reactions, the organism was classified as Pasteurella pneumotropica, a classification that was confirmed by the Communicable Disease Center, Atlanta, Georgia.

P. pneumotropica was first isolated and characterized by Jawetz.^(7,8) He showed that the organism was commonly latent in apparently normal animals and that it was difficult to demonstrate pathogenicity without serial passage through animals. Jawetz and Baker⁽⁹⁾ described the pathology and pathogenesis of the lesions associated with infections with virulent strains of this organism.

Hoag, et al.⁽¹⁰⁾ studied latent infections of P. pneumotropica in a mouse colony and isolated the organism on 81 occasions from numerous tissues in several strains of mice. They were unable, however, to demonstrate any latent virulence or pathogenicity in naturally or experimentally infected animals.

The bacteria isolated and studied by both Jawetz and Hoag et al. were biochemically identical. The one we isolated differs, however, in that it does not ferment mannitol.

Heyl⁽¹¹⁾ also described an organism isolated from mice with pneumonia, which he classified as P. pneumotropica. His strain, too, consistently failed to ferment mannitol.

Since our studies on P. pneumotropica were begun, we have isolated the organism 67 times from a number of species of animals, including six different strains of mice. These isolates were obtained from a variety of tissues and in some cases from several tissues of the same animal. The most common occurrence has been in the lungs of mice and rats (21 cases), and in each case the organism was associated with pneumonia. The second most common source was the eyes of mice (16 cases); in each of these cases conjunctivitis was present. Other sources of the organism have been uteri and vaginas of mice with metritis, local abscesses (peritoneal and pleural) in mice, the ear of a dog with otitis and the lung of a hamster with pneumonia.

Our isolation of the organism from the eyes of mice affected with conjunctivitis is the first reported from this organ. Nelson⁽¹²⁾ studied conjunctivitis in a colony of mice and found that pleuropneumonia-like organisms (PPLO) were commonly associated with the disease. However, his attempts to reproduce the disease by direct contact or by installations of

ocular washings from affected mice were largely unsuccessful. His observation that conjunctival smears of affected mice commonly contained numerous gram-negative bacilli suggests that perhaps a synergistic reaction between two organisms or other predisposing factors are necessary for the onset of clinical signs. It is also possible that the gram-negative bacilli that he failed to identify were P. pneumotropica.

Several attempts to produce pneumonia or conjunctivitis with a newly-isolated strain of P. pneumotropica, including one attempt that involved stressing the animals by placing them in a 4°C environment, have been unsuccessful. The organism is now being passed in 18 to 21-day-old mice in an attempt to increase its virulence, as described by Jawetz.⁽⁷⁾ The possibility that P. pneumotropica might react with or require another organism in order to exert its pathogenicity is also being investigated.

It should be pointed out that our studies of this organism thus far have not been of the nature of a survey. In each instance, the isolation of the organism was attempted because of clinical evidence of disease. We hope to conduct a survey in the near future to determine the incidence of this bacterium in apparently normal animals, and we expect to find that it exists in a high proportion of them.

IV. Hemorrhagic Diathesis in Laboratory Rodents

Thomas E. Fritz, David V. Tolle,
Robert J. Flynn, and Calvin M. Poole

The ever-present problem of chronic infections in laboratory rodents, particularly infections of the respiratory tract, has prompted us to develop breeding colonies free of these infections.

Fifty-four pairs of specific-pathogen-free rats, initially raised germ-free until weaning age, were obtained from a commercial breeder. These were set up in breeding pairs at 12 weeks of age. Each pair successfully conceived a first litter with very few losses among the young and none among the adults.

When the rats were about 16 weeks of age, one of the males died of prolonged hemorrhage from a minor laceration. Within the next four weeks, another 30 of the males had died, and within eight weeks 44 of the 54 were dead. During this same period, three females also died.

Most often the animals were not noted as sick but were found dead in their cages. Those observed to be sick showed generalized anemia, hemorrhage from the conjunctiva and external nares, hemorrhage of the skin of the

face, subcutaneous hemorrhages or hematomas in various areas (but predominantly in the cervical area), hemorrhage into the tunica vaginalis visible through the scrotum, and in several instances posterior paralysis.

The animals with posterior paralysis consistently had extensive hemorrhage of the brain, the spinal cord, or both, but hemorrhage in these areas was also observed in animals without nervous involvement.

Other lesions observed at necropsy included mild to marked hepatic degeneration, and hemorrhages in the scrotum, the pleural cavity, the pelvic and perirenal peritoneum, and the ventral cervical area.

Coincidental to, and beginning almost simultaneously with, the hemorrhagic disease in the rats has been the occurrence of a similar syndrome in our colony of specific-pathogen-free mice. This colony was initiated by obtaining litters by cesarean section and foster-nursing them on germfree dams. The animals used to start this colony consisted of 26 females and 13 males.

When these mice were about 10 weeks of age, four of the males died. Within another 10 days the remaining nine males died. The signs shown previous to death included dyspnea, depression, and generalized anemia. The most consistent single lesion was a marked bilateral hemothorax.

A few days after the last of the original males had died, one of the females died with similar signs preceding death. At necropsy the significant finding was the presence of extensive hemorrhage into the uterus, which had been gravid.

Since the death of this first female, approximately 25 others have died. In each instance the signs of anemia were prominent before death if the animal was observed to be sick at all, and in each instance extensive hemorrhage into the gravid uterus was present. No nonpregnant female has been involved.

Meier et al.(13,14) reported a hemorrhagic diathesis in mice, which they related to the ingestion of glycols formed in bedding sterilized with ethylene oxide. That our problem might be due to a similar intoxication was supported by the fact that the original purchased rats were raised on a type of bedding that at one time was supplied presterilized with ethylene oxide. Similarly, the mice we used as a nucleus for our colony were raised on the same type of bedding. The manufacturer of the bedding has assured us, however, that none of our supply was treated with ethylene oxide and that it is very unlikely that the bedding of the breeder from whom we purchased our rats was so treated.

We are presently having our bedding analyzed for glycols. Should it be found glycol free, the work of Meier et al. will need to be reevaluated. Even though they were able to reproduce their syndrome experimentally by gavage with glycols, they may have been greatly simplifying a complex syndrome. They did show, however, that in the diathesis they studied there were prothrombin-complex deficiencies, specifically deficiencies of factors II, VII, IX, and X.

Our laboratory tests also suggest deficiencies in factors II, VII and X. Yet our syndrome differs from that of Meier et al. in that our second generation mice, born and raised on bedding that has had no chance of being contaminated with glycols, are also dying. This is also true of our second generation rats.

Malhotra⁽¹⁵⁾ described a similar hemorrhagic syndrome in rats. He demonstrated that administration of vitamin K and methionine to affected animals prevented spontaneous hemorrhage, but he did not establish the relationship of methionine to prothrombin or show how the several other essential factors might be influenced.

Barnes and Fiala⁽¹⁶⁾ reported the rapid development of vitamin K deficiency in rats receiving vitamin K-free diets when coprophagy was prevented, and Gustafsson⁽¹⁷⁾ demonstrated the occurrence of a vitamin K deficiency and subsequent development of a hemorrhagic disease in germ-free rats. Since both our affected rats and mice originated in a germ-free environment, the intestinal flora or, rather the lack of it may be an important predisposing factor. This is now being studied.

V. Pneumonia Epizootic in Mice

Robert J. Flynn, Thomas E. Fritz,
Patricia C. Brennan,
and Calvin M. Poole

During the period covered by this report, an unusually high incidence of pneumonia was observed in one animal room. This room contained about 750 CF#1 mice, 10 to 12 months old, until May 1963, when a breeding colony of about 800 AX mice up to 3½ months of age, were moved in with them. Pneumonia had been previously observed in the older CF#1 mice, but until this time, it had not been noted in the AX mice.

In July, two of the AX mice, which then numbered 1900, were found, both grossly and histologically, to have pneumonia. Another case occurred around the middle of August. Thereafter numerous cases were observed and

both the AX and CF#1 groups were culled heavily. Many mice from both strains were necropsied. All the CF#1 mice examined showed varying degrees of pneumonic involvement; most were severely affected. There was also a high incidence in the AX mice examined. No significant gross lesions were observed in any other organs or tissues in either group except for secondary involvement of the lymph nodes of the thoracic cavity.

Histologically, the most consistent and conspicuous feature was the formation of extensive peribronchiolar lymphoid aggregates. The extent of involvement varied from animal to animal, but in general the CF#1 mice had a much greater portion of the lungs involved and the features of the reaction suggested it to be of a longer duration.

Attempts were made to isolate the causative agent. Lungs from 12 AX and 9 CF#1 mice were cultured individually and then pooled and stored frozen for future studies. Pasteurella pneumotropica was recovered from most of the specimens. Pleuropneumonia-like organisms (PPLO) were not recovered initially because of technical difficulties, but they were later recovered from one AX mouse.

Mice known to be free of all common pathogens were inoculated intranasally with bacteria-free filtrates of a homogenized suspension of the frozen, pooled lungs, but we were unable to reproduce the disease.

Studies are now under way to test the effects of inoculating filtrates of homogenized suspensions of fresh pooled lungs alone and in combination with P. pneumotropica, PPLO, or both.

VI. Rodent Breeding Program

Thomas E. Fritz, Robert J. Flynn,
and Merlin H. Dipert

A method for maintenance of breeding records that utilizes the computer facilities of the Division is being developed. Data now being recorded from the Division's production colonies include daily observations on all changes in the health status, births, deaths, weanings, weaning weights, cullings, and the issuance of animals for experimental use. These data are analyzed weekly, and a summary is prepared that lists the number of litters born, the number of animals born, the average litter size, the number of young animals dead, sick, culled, or missing, the number of litters weaned, the number of animals weaned, the sex distribution of the animals weaned, the average litter size weaned, the total breeding pairs presently in production and the number of breeding animals sick, dead, culled, or missing.

It also includes a list of animals that will be of weaning age during the next week and thus relieves the technical assistants of the job of calculating the age of each individual litter on each weaning day.

This program will provide a continuous critical analysis of the breeding efficiency of the colonies and will also provide a continuous check on the health status of animals intended for experimental use. It is believed that these procedures are unique in the breeding of laboratory animals and that they will prove to be of great usefulness in providing experimenters with well-defined laboratory animals.

VII. Dog Care Program

Calvin M. Poole and
Robert J. Flynn

A program to provide professional veterinary care for all the Division's dogs and clinical support for the investigators using these animals was initiated during the fall of 1963. Specifically the program provides daily observations of all dogs; canine distemper-hepatitis vaccinations at 12 weeks of age, 4 months of age, 1 year of age, and annually thereafter; routine physical examinations every 4 months; routine dental examinations at 1 year of age and every 4 months thereafter; parasitological examinations at 6 weeks of age, 10 weeks of age, and every 4 months thereafter; examination, diagnosis, and treatment of all observed diseases or abnormalities; professional surgical assistance and postoperative care; routine care of newborn pups. A typical month's activity is shown in Table 6.

TABLE 6

Dog care activities for a typical month
(December, 1963)

Anesthetics given:	121
Routine dental examinations:	109
Routine physical examinations:	135
Distemper-hepatitis vaccinations:	90
Treatments of eye conditions:	23
Treatments of ear conditions:	11
Individual treatments for internal parasites:	145
Pregnancy and pre-partum examinations:	19
Miscellaneous treatments or examinations:	74
Surgical procedures (cesarean sections):	2

In general the health status of the dog colony is extremely good. There have been no contagious diseases nor external parasites observed during this period. The incidence of internal parasitism has not yet been completely determined; however, as of this date only coccidia and one species of roundworm, Toxascaris leonina, have been observed.

As a part of this program, studies are being made of the relationship of dental calculus to periodontal disease and of factors affecting and methods of preventing calculus formation.

VIII. Use of an Ultrasonic Unit in the Dental Care of Research Dogs

Calvin M. Poole and Robert J. Flynn

Cleaning dogs' teeth and removing dental calculus by hand is laborious and time consuming. For this reason we have procured a Dentsply-Cavitron Unit, Model 30 C.*

The unit operates by converting ordinary 50 or 60 cycle AC current into 25,000 cycle current. This in turn is converted into 25,000 small (0.001 in.) mechanical strokes per second. These strokes are transmitted to the teeth via a hand piece fitted with a prophylaxis tip and accompanied by a water spray. In addition to the mechanical strokes, cavitation action is produced in the water.

The dog to be treated is anesthetized with an ultra-short-acting barbiturate and the scaling is accomplished by guiding the prophylaxis tip over the exposed teeth and subgingival areas, wherever calculus and debris have accumulated, in the same manner as writing with a ball point pen. It is not necessary to apply any more pressure than one would in writing, since the vibrations and cavitation, rather than pressure exerted by the operator, accomplish the scaling. There is minimal hemorrhage associated with the use of this instrument and no apparent soft tissue damage. The water spray prevents overheating, serves to aid the cavitation action, and flushes away blood and debris.

The unit not only greatly reduces the scaling time and effort but gives better results than traditional hand scaling. The use of this type of instrument in human dentistry has been increasing in popularity. It is also being used occasionally in veterinary medicine. However, we believe this is the first report of its use in the care of research dogs.

* Cavitron Ultrasonics Inc., Long Island City 1, New York.

IX. Dog Breeding Program

Calvin M. Poole and
Robert J. Flynn

The present breeding colony consists of beagles derived from stock procured from the University of Utah, the University of California (Davis), Cass O'Day Kennels (Murray, Utah), and Dr. James W. Caden (Alexandria, Alabama).

For controlled, reproducible research, the animals used should have a minimum of variation. Therefore, the dogs should be of a fixed strain, within a specified size range, healthy, free of anatomical or physiological defects and docile in temperament. The objective of the breeding program is to produce beagles that meet these qualifications and to produce them in numbers sufficient to satisfy the experimental needs of the Division.

After reviewing the advantages and disadvantages of inbreeding and outbreeding and comparing the expected results from each, we feel that a modified inbreeding program (closed line or family) would more nearly approach the desired goal. Because the number of available inbred animals is insufficient and because of the uncertainties associated with any inbreeding program, some outbred production will be continued.

The two existing inbred lines, the "Blondes" line and the "Alabama" line, will be continued. However, sibling matings will be limited in number and the majority of matings will be more distantly related, not closer than first cousins if possible. It is realized that this type of breeding does not rapidly increase the inbreeding coefficient but it does decrease the possibilities of encountering difficulties in maintaining the lines.

As of December 31, 1963, the breeding colony consisted of 28 outbred females and 2 outbred males; 20 inbred females and 8 inbred males of the Alabama line; and 5 inbred females and 4 inbred males of the Blonde line.

The dog production from July 1, 1963 to December 31, 1963 consisted of 117 pups in 20 whelpings, giving an average litter size of 5.8 pups. Twenty-nine of the 117 pups died. Most of these deaths were stillbirths, neonatal or accidental, and did not suggest any serious breeding or disease problem. It is of interest to note, however, that of the 20 whelpings only 2 were from inbred matings. This can be accounted for in many ways and is of no great significance at this time. It does indicate, however, that the productivity of the inbred animals will have to be carefully observed and evaluated in the future.

X. New Strains and Species: Japanese Quail (*Coturnix coturnix*)

Durward D. Banister, Calvin M. Poole,
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The coturnix quail is a small game bird that occurs naturally in most parts of the world outside the Americas. It resembles our native quail in size and general characteristics. Padgett and Ivey(18) and Howes and Ivey(19) have suggested the use of the Japanese variety in research. Because of interest expressed by some members of this Division, it was decided to obtain some and to study their care and breeding requirements and possible usefulness.

In May 1963, 100 males and 100 females were obtained from Auburn University. These and their subsequent progeny were housed in standard (24 in. x 36 in. x 9 in.) chicken brooders. This type of housing appeared to be quite satisfactory for all but newly hatched chicks. The adult females were housed in groups of 20-25, but we quickly found that the males had to be housed individually, except when they were put with the females, to prevent them from killing each other.

Japanese quail mature quite rapidly and start laying at 45-50 days of age, with full production, about one egg every other day, by 70 days of age. We have incubated approximately 400 eggs at 100°F and 60% relative humidity in a regular automatic incubator of the type used to hatch chicken eggs. Because of the small size of the quail eggs, it was necessary to put them in the incubator in special wire tubes to keep them and the newly hatched chicks from falling through the incubator tray openings. We found that eggs from hens 70 days old or older had a hatchability of 50-54% while those from hens that were 45-50 days old had a hatchability of only 4-6%. We also found a lowered hatchability in eggs kept at room temperature for more than five days. The incubation period varied from 15 to 18 days, with the majority hatching on the 17th day.

As the chicks hatched, they were removed from the retaining tubes, placed in small plastic containers, and returned to the incubator for another 24 hours. At the end of this time, they were removed from the incubator and put into improvised brooders consisting of standard ANL plastic rat-cage bottoms (23 in. x 11 in. x 6½ in.) heated with light bulbs or heat lamps. Initially 10-20 one-day-old chicks were placed in each plastic cage bottom. Paper towels were used as bedding, and a pint Mason jar fitted with a plastic screw-on water trough was used for watering. The chicks were fed chicken starter-grower mash from a shallow petri dish.

When they were 9 to 10 days old, they were rather well feathered and the heat source was removed. At about 14 days of age, covers had to be placed on the plastic cage bottoms to keep them from flying out. Because of their rapid growth rate, they had to be separated rather often to avoid overcrowding. At 25-30 days of age they were placed in the regular chicken brooders, 20 per brooder (16 females to 4 males). They were continued on chicken starter-grower mash and appeared to do quite well.

Sex can be determined by size and breast feather coloration. The female usually weighs about 125-175 g and has a grey and black speckled breast that is lighter in color than that of the male. The male usually weighs about 105-135 g and has brown or rust-colored breast feathers, with fewer speckles than the female.

Clinical evidence of disease other than occasional feather picking was notably absent. Only one case of illness was observed, an adult female that exhibited torticollis, incoordination, loss of equilibrium and rolling to the right when recumbent. Chronic septic meningitis and osteitis were observed at necropsy.

To determine the presence of chronic or subclinical diseases in the flock, approximately 180 of the original adult birds were killed and necropsied. No significant gross lesions were observed, nor were any internal parasites found by either direct or microscopic examination. Serum samples from five birds were tested for Newcastle disease, pullorum and PPLO infection. All were negative.

In summary, the Japanese quail is relatively easy to raise, apparently can be produced the year round, matures rapidly, is very hardy, and requires much less space than the chicken or other commonly used laboratory birds.

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TOXICITY AND METABOLISM OF RADIONUCLIDES

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Pathologic Alterations in Beagle Dogs Injected Intravenously with Cerium-144 at the 50 $\mu\text{C/kg}$ Level*

Experiment 003

One dog (0012) died 955 days after injection with 50 $\mu\text{C/kg}$ of cerium-144.

Gross Pathology

The animal was dehydrated. Decubital ulcers occurred over the lateral skeletal prominences. The musculature of the posterior limbs was atrophic. An osseous tumor involved the body of the second thoracic vertebra and extended into the first vertebra (Figure 8). The vertebral ends of the first and second ribs, right and left, were incorporated within the tumor mass for a distance of 1 cm or less. Due to extension of the tumor mass into the vertebral canal, there was constriction and atrophy of the spinal cord at the level of the first and second thoracic vertebrae. Longitudinal sectioning of the long bones revealed coarse trabeculation.

Figure 8

Sagittal section of first four vertebrae of dog 0012. The osteogenic sarcoma arises from the second thoracic vertebra and extends into the vertebral canal.

Histopathology

The tumor of the thoracic vertebrae was an osteogenic sarcoma. The growth was made up of cartilage, osteoid, bone, and soft tissue, in which round and spindle cells were numerous (Figure 9). The masses of

*This work was performed by Louise S. Lombard.

bone and cartilage were rudimentary and irregular. Radioautographs showed both beta aggregates and alpha tracks in the area of the tumor as well as in the bone marrow and compact bone of the long bone, vertebrae, ribs, and sternum.

Figure 9

Histologic appearance of tumor shown in Figure 8. There are areas of bone, osteoid and cartilage interspersed among areas of spindle-shaped sarcoma cells. (Giemsa)

Pathologic Alterations in Beagle Dogs Injected Intravenously with Cesium-137 at the LD_{50/30} Level

Experiment 004

One dog (0441) died 52 days after injection of 2704 $\mu\text{c}/\text{kg}$ of cesium-137, while another (0449) was euthanized on the 38th day after injection of 2650 $\mu\text{c}/\text{kg}$, as part of a sacrifice series.

Gross Pathology

Dog 0441. The animal was markedly dehydrated. The lungs were pale pink and contained multiple petechial and ecchymotic hemorrhages in all lobes. The heart blood was pale pink and watery. The pericardial sac was thickened by edematous fluid and the myocardium was streaked with multiple linear hemorrhages. Mucous membranes, conjunctivae, and

visceral organs were blanched. The cortex of the adrenal gland was pale pink with cream-colored streaks in the mid-cortical zone; the ratio of cortex to medulla was 3 to 1. The cortex of the kidney was streaked with cream-colored linear markings. Petechiae appeared throughout the brain. The bone marrow of the long bones was fatty throughout the length of the shaft and contained sparse small red foci (Figure 10). The spleen was fibrotic. Lymphoid tissue of the spleen and lymph nodes was hypoplastic.

Figure 10

Section of femur of dog 0441. The marrow is cream-colored and fatty throughout the cavity and contains scattered reddish-purple foci.

Dog 0449. Hemorrhages of varying sizes occurred within the ventral cervical muscles, in the heart, small and large intestine, kidneys, thymus, gall bladder, glans penis and testes. The bone marrow was a bright reddish-purple.

Histopathology

Dog 0441. The bone marrow was aplastic with the exception of small areas which contained aggregates of dividing cells. Myelopoietic foci were numerous and erythropoietic foci comparatively sparse (Figure 11). Contrast radioautographs of the femur indicated a concentration of radioactivity along the endosteum and in areas containing actively dividing cells. The adrenal cortical lipid was limited to patchy linear areas within the zona fasciculata. Alterations within skeletal muscle consisted of a slight thickening of the sarcolemma due to glycogen infiltration.

Figure 11

Histologic appearance of the bone marrow of dog 0441. A small nest of cells, including plasma and reticulum cells, osteoclasts, and degenerating megakaryocytes, lying along the endosteum. (Giemsa)

There was vascular degenerative change and thrombotic involvement of the venules and capillaries of the heart, lungs, skeletal muscle, brain and liver. The thrombi consisted of fibrin, lipid and unidentified hyaline-like material. These thrombi were either fibrillar, hyaline or vacuolated and stained metachromatically with Giemsa, were positive following periodic acid-Schiff (PAS) treatment of diastase-digested material, positive for the SH radical (dihydroxydinaphthylidissulphide test), and stained blue with phosphotungstic acid hematoxylin. The fibrillar thrombi stained blue with Weigert's fibrin and phosphotungstic acid hematoxylin. The vascular lesions consisted of thickening of the vessel wall with a hyaline-like material and with extravasation of erythrocytes within and about the vessel wall.

Dog 0449. The bone marrow was hypoplastic (Figure 12); all long bones and ribs contained foci of granulopoietic and erythropoietic cells. Mitotic figures were noted along the endosteum. The Malpighian corpuscles within the spleen and lymph nodes were small and sparse, and there were no megakaryocytes. Mitotic figures were rare in the spleen and numerous within the lymph nodes where abnormal mitotic figures were observed. Approximately one-third of each lymph node was replaced by adipose tissue. The skeletal muscles contained increased concentrations of glycogen within the sarcolemma (Figure 13).

The testes were atrophic. Only rare tubules contained primary spermatocytes, while spermatozoa, Sertoli cells and spermatogonia were

present in most tubules. Mitotic figures were rarely observed. Multinucleated giant cells appeared free within the lumen of the tubules.

Figure 12. Histologic appearance of the bone marrow of dog 0449. The marrow contains differentiated cells, necrotic cells and mitotic figures. (Giemsa)

Figure 13. Cross section of the temporal muscle of dog 0449. The glycogen infiltration appears as a thick cuff between the sarcolemma and the muscle fiber. (Periodic acid-leucofuchsin-Schiff)

The venules throughout the body were thickened by a subendothelial hyaline-like material. Extravasations of erythrocytes appeared within or about the vessel wall. The venules contained thrombi similar to those described for dog 0441.

Control Dogs

Dogs of different ages were sacrificed for control tissues. The four dogs listed in the present report were: numbers 0049 (66 months); 0325 (31 months); 0405 (25 months); and 0463 (15 months).

On gross examination it was found that dog number 0049 had chronic valvular endocarditis of both atrioventricular valves. Microscopic findings included the observation that glycogen infiltration of the sarcolemma was present. This change was similar to that observed in the experimental dog 0449.

Dog 0325 received 7 mc of tritiated thymidine two hours before being euthanized with nembutal. The tissues collected from the dog were used for a study of cell renewal rates in all major organs, in addition to being used for histologic study. No gross or microscopic abnormalities were observed.

Dog 0405 was considered normal on gross and microscopic examination.

Dog 0463, previously used for K⁴⁰ tracer studies, had clinically observed heart murmurs. On gross examination, a congenital ventricular septal defect was found. The aortic valve leaflets had mineralized annular thickenings at the free margins. The septal defect occurred beneath the aortic valve. The left ventricle was dilated, and the atrioventricular valves had fibrous, nodular thickenings on their free margins. The lungs contained multiple fibrotic areas in association with bronchiectasis.

Stock Dogs

The causes of death in the nonradioactive dogs on standard and semisynthetic diets are listed in Table 7.

TABLE 7

Causes of death in nonradioactive dogs on standard and semisynthetic diets

Cause of death	Number of dogs	
	Standard diet	Semisynthetic diet
<u>Adult</u>		
Uremia, urethral obstruction with calculi		1
<u>Young</u>		
Septicemia*	1	1
Bronchopneumonia	2	1
<u>Newborn</u>		
Pneumonia, hemolytic <u>Streptococcus</u>	2	
Birth injury	1	
Septicemia*	4	
Peritonitis, umbilical infection	2	
<u>Stillborn</u>	4	

*In those animals in which cultures for bacterial examination were prepared a beta hemolytic Streptococcus was found.

Pathologic Alterations in Sprague-Dawley Rats Injected
Intravenously with Cerium-144 at the LD_{100/30} Level

Pilot studies were undertaken with cerium-144 in rats to develop technics for estimating the degree of damage inflicted upon the reticulo-endothelial system by high dose levels of cerium-144. Table 8 lists the animals by number, dose level, and days to death.

TABLE 8

The acute toxicity of cerium-144 in Sprague Dawley rats

Number	DL-ethionine, g	Ce ¹⁴⁴ injected, μ c	Ce ¹⁴⁴ dose level, μ c/g	Proferrin injected, mg	Survival after injection, days	
					Ce ¹⁴⁴	Proferrin
1 (control)*	0	0	0	0		
2	0	640	5	0	8	
3	0	1000	5	0	9	
4	0	715	4	0	11	
5	0	663	4	0	12	
6	0	715	4	0	12	
7	0	663	4	0	11	
8	0	715	4	0	11	
9	0	848	4	14.2	8	3
10	0	904	4	15.1	9**	5
11	0	840	4	0	3†	
12	0	650	3	19.5	15**	2 hrs
13	0	0	0	17.0		2 hrs
14 ††	1.5	0	0	0		
15	1.5	884	3	0	5**	
16	1.5	723	3	0	5**	
17	0	845	3	0	5**	
18 (control)*	0	0	0	0		

*Euthanized 12 days after initiation of experiment.

**Euthanized with nembutal.

†Died following liver biopsy.

††Euthanized rat 14 on 25th day after DL-ethionine treatment was initiated.

The rats were injected via the lateral caudal veins while under nembutal sedation. Rats pretreated with DL-ethionine were given a 0.5% solution of DL-ethionine in their drinking water for 20 days before injection with cerium-144. Rats which received iron dextran (Proferrin)* were injected with 6.7 mg Proferrin/100 g of rat. Tissues were taken for histological section and prepared for radioautographs as described previously

*Proferrin (saccharated iron oxide) contains the equivalent of 20 mg of available iron and is manufactured by Sharp and Dohme, Philadelphia, Pennsylvania.

for canine tissues.⁽¹⁾ Liver biopsy was attempted in one rat (No. 12) using the Müller biopsy needle; however, the animal succumbed within one hour following surgery.

Prior to injection, the cerium-144 solution was examined for degree of ultrafilterability using the technic of Lindenbaum.⁽²⁾ The injection solutions of cerium-144 were found to be 90 to 94% nonfilterable.

Gross Pathology

The rats which died showed the following lesions. Bloody fluid was noted about the nares and anus. Hemorrhages occurred in the subcutis and dermis, in the heart muscle, lungs, stomach and intestines; there was congestion of the bone marrow, liver and kidneys. Necrotic foci were scattered throughout the liver.

Figure 14. Liver of rat 4. Wedge-shaped areas of hepatic cell necrosis occur above and below the central vein. In the necrotic areas, many reticuloendothelial cells remain. (Hematoxylin and eosin)

Histopathology

Cerium-144 Group

Liver. Focal areas of hepatic cell necrosis occurred in the liver of all rats (Figure 14). Bacterial emboli were observed only in those animals that died. Emboli were associated with large areas of hepatic cell necrosis. In such lesions, hepatic cell cords had undergone coagulation necrosis while the reticuloendothelial cells remained intact. Upon radioautographic examination, it was noted that numerous aggregates of beta radioactivity occurred in reticuloendothelial cells of the liver. Diffuse radioactivity appeared in hepatic cells about the central veins.

Bone Marrow and Bone. The bone marrow was aplastic (Figures 15 and 16). Cells remaining within the

bone marrow consisted of fat cells, mature erythrocytes, mast cells and phagocytes. In the rats killed at 5 days, occasional differentiated granulocytes were seen. Mast cells were considerably more numerous in the animals receiving cerium-144 than in the control rats. The mast cell granules varied considerably in size and staining characteristics. Large,

round phagocytic cells were prominent; within their cytoplasm there were as many as 20 erythrocytes (Figures 17 and 18). Radioautographs indicated that beta radioactivity was within the phagocytes in bone marrow, along the endosteum of trabecular and compact bone, and in blood capillaries within the epiphyseal zone.

Figure 15. Femur of rat 6. The bone marrow above and below the epiphyseal line is aplastic. (Giemsa)

Figure 16. Higher magnification of the femur shown in Figure 15. The bone marrow in the metaphysis consists mainly of loose mesenchymal tissue. (Giemsa)

Spleen and Lymph Nodes. The lymphoid tissues were hypoplastic or aplastic. The radioactivity within the spleen was heaviest in the perifollicular region, whereas in the lymph nodes the activity was concentrated in the subcapsular sinuses.

Kidney, Lung, Gonads, Endocrine Organs, Duodenum and Colon. Diffuse radioactivity was observed in the epithelial cells of the convoluted tubules and in the glomerular tufts of the kidney; in addition, aggregates of radioactivity occurred within phagocytic cells in the interstitium.

Aggregates of beta radioactivity appeared in the alveolar lining cells, in phagocytic cells lying free within alveoli, and in phagocytic cells within the interstitial tissue.

Figure 17. The femur of rat 5. The sinusoids of the shaft are distended with erythrocytes. Erythrophagocytes are numerous. (Giemsa)

Figure 18. Higher magnification of Figure 17. Mast cells (M) are interspersed within the mesenchymal tissue of the marrow. Phagocytes (P) contain large numbers of engulfed erythrocytes. (Giemsa)

Aggregates of beta radioactivity were noted in the phagocytic cells of the gonads, the endocrine organs, and the digestive tract.

Cerium-144 and DL-Ethionine Group

The findings were similar in all organs to those seen in rats receiving cerium-144 alone, with the exception that, in addition, the livers of the DL-ethionine-treated rats contained a few bizarre mitotic figures and areas of bile duct proliferation (Figure 19).

Cerium-144 and Saccharated Iron Oxide Group

The distribution of saccharated iron oxide was widespread throughout the reticuloendothelial system; however, it was most abundant in the spleen and liver. Cells containing beta radioactivity also contained saccharated iron oxide.

Figure 19

Radioautograph of the liver of rat 15. Diffuse and aggregated beta radioactivity appear in the liver. In the right field there are two areas of bile duct proliferation (BD). (Hematoxylin and eosin)

Iodine and Thyroid Metabolism*

The following is a progress report of work being carried on in which the effects of I^{131} on thyroid function will be studied. This report covers the period from July 1 to December 31, 1963, and represents initial exploratory experiments designed to give general information regarding techniques which will be useful in the later study of irradiated animals.

The first objective of the new program was, and still is, to measure changes in thyroid function in dogs that have been given I^{131} alone or with another isotope, such as Cs^{137} or Ce^{144} . It is possible with existing methods to determine extreme states of hyper- or hypoaactivity of the thyroid. Between these extremes there is a continuum of functional activity which may fluctuate in response to many varied physiological changes. It is in this area of subtle change that one encounters the greatest difficulty in assessing thyroid function. However, it is precisely in this area that the most revealing information regarding the nature of these changes must and will be gained. The following exploratory experiments have been performed with the hope that the insight gained might lead to a refinement of techniques for measurement and assessment of thyroid function. Several of the procedures to be outlined have been performed on single animals for a number of reasons, not the least of which is the limited supply of available

*This work was performed by Robert J. Toft.

dogs, and the data are necessarily fragmentary and not always entirely revealing. They do, however, give indications of the probable success or failure of a particular technique or approach and for that reason are valuable.

Short-term Uptake of I^{131} by Thyroid

Larsson and Jonnson⁽³⁾ described a method for determining thyroid function based upon the uptake of I^{131} by the thyroid within the first 15-20 minutes after intravenous injection. A fast rising rate of uptake was indicative of hyperthyroidism while a gradual diminution in count rate over the thyroid after an initial rapid rise indicated hypothyroidism. A modification of this technique was attempted with dog number 0570. The dog was injected intravenously with 1.4 ml (75 mg) of promazine hydrochloride (Sparine) in the right cephalic vein. This was followed by an injection of 2.5 ml (125 mg) of sodium pentothal. After being anesthetized the dog was suspended in a sling, constructed of plastic and tape, which allowed the legs to hang in a normal standing position while the body of the animal was supported. The dog was suspended over a table on which was placed a $1\frac{5}{8}$ in. x $1\frac{5}{8}$ in. sodium iodide scintillation crystal. The crystal was positioned under the area of the thyroid $3\frac{1}{2}$ inches from the surface of the neck. A 400 channel RIDL analyzer programmed for sequential counting with an interval of 10 seconds per channel was attached to the crystal-photomultiplier-preamplifier unit.

The analyzer was started and background counts were stored in several channels before the syringe containing the I^{131} was brought into the area. The injection was made in the left cephalic vein. A sequential count was taken over the thyroid area for approximately 20 minutes after the injection. The amount of I^{131} injected was determined by measurement of the syringe both before and after the injection with a 4 in. x 8 in. sodium iodide crystal in the iron room.

The data from the injection solution and residue indicated that dog 0570 received 4.95 microcuries of I^{131} ($0.45 \mu\text{c/kg}$). When the data from the uptake were plotted, a positive slope was noted. It was, however, very slight and would be considered to be in the euthyroid range according to the criteria of Larsson and Jonnson.⁽³⁾ There was noted a disturbing oscillation in the counts from channel to channel. These oscillations suggested that there might be some systematic malfunction in the machinery. When a pulse generator was coupled with the scanning device, it was found that the counts varied not at all from channel to channel. The answer was then sought in the data itself. A program for the IBM 1620 computer was devised which would average several channels, drop the first, add the next, and give a running average which would tend to smooth the curve and eliminate some of the extremes, thus enabling one to see better the nature of the oscillations if they still existed. After some discussion it was

decided that the oscillations noted were random and were caused by the small number of counts in each channel. Data with oscillations similar in nature but different in period were obtained using a variety of standards, proving that the noted event was not in any way connected with the peculiarities of the uptake of I^{131} by the thyroid.

Because of the problem of quantitating the degree of the slope with any particular physiological function and because the arrangement for measuring the thyroid was not yet standardized, it was decided to postpone further excursions into this method until a later date. A series of experiments is now being planned which will once again incorporate this design with many refinements and will, we hope, give more insight into the relationship between the speed of uptake and the physiology of the thyroid gland.

After the initial measurement of uptake the dog was measured daily in the iron room using the sling arrangement and the 4 in. x 8 in. crystal with the 400-channel analyzer in pulse height mode. Because of inexperience with this type of measurement the dog was not measured immediately after the iodine injection, and so no measure of initial absorption was obtained. This has been corrected in subsequent injections with a couple of exceptions. The initial whole-body measurement which was made one day after injection showed a biological retention of 63% of the injected dose. This figure represents the amount remaining after 24 hours during which time a considerable amount may have been excreted in the urine as will be noted later. It is also in error by the amount which the absorption by the animal's own tissues prevents the radiation from reaching the crystal. Later studies have indicated that this absorption factor may represent a 25-35% loss of counting rate with an intravenously injected dose and as much as a 50% loss immediately after oral ingestion of a capsule containing the I^{131} . Future work includes plans for a complete study of the geometric and absorption factors. The analysis of the data by the computer is such that these factors may be included at any time and a recomputation made of the true biological retention. It should be borne in mind that all subsequent data to be presented suffer from the same lack of correction for absorption. The absolute values to be presented are, therefore, not correct; however, the relative comparisons are valid.

There was a definite break in the retention curve at about the eighth day when the loss of iodide became much less. An increase in retention noted at day 20 can only be explained by an error in the technique of counting wherein the geometry of the counting setup must have changed.

Whole Body Retention - Preliminary Measurement

Following the work with 0570 three more dogs were given I^{131} for a study of whole-body retention of the isotope. The first two of these, 0078 and 0181 were injected intravenously with I^{131} as was 0570. Number 0233

was given a capsule orally containing the isotope. Dog 0181 was measured at intervals for 1 week, while the retention in the 2 other dogs was followed for 2 weeks. The four dogs all exhibited similar patterns of retention. There was an initial rapid decrease in retention with a change in the rate occurring about the sixth to eighth day after injection or ingestion of the isotope. The initial level varied considerably from one dog to another. In each case the leveling off of the rate of excretion came at about 6-8% retention. It is unfortunate that dog 0181 was not measured for a longer period of time. There is evidence that at the time of the last measurement which was made at 7 days that the curve had begun to flatten as in the other 2 dogs. One can only speculate as to whether or not it would have attained the same degree of slope as did the other dogs after eight days.

Thyroid Retention of I^{131} Apparatus for Measurement

Because of reports in the literature that the rate of release of iodine from the thyroid was a sensitive measure of thyroid activity a renewed interest was taken in thyroid measurements. An aluminum collar was designed which would fit on to the lead shield surrounding the crystal used for thyroid measurements. It was so designed and constructed that the neck of the dog could be centered over the crystal in a reproducible fashion. There are "wings" projecting upward from the collar on either side of the crystal which serve to prevent the dog from either turning his head or from moving his neck from side to side. The anterior portion of the collar rests just posterior to the angle of the jaw while the posterior end of the collar is just in front of the collar bone. In this way the animal can be held in a fixed position for the five-minute period during which a count is being made. It is necessary only for an assistant to hold his hand on the top of the dog's head gently to restrain him. The whole crystal assembly is mounted on an unistrut frame which can be elevated. The assembly can be moved back and forth on the frame so that any position may be achieved with the crystal. The crystal assembly is further made to rotate on an axis perpendicular to the plane of elevation. The whole apparatus is mounted on a cart for ease in moving from one location to another. A dog is placed on the top of the cart and allowed either to sit or to stand. The crystal assembly with the aluminum collar is then elevated, advanced, and tilted as necessary to fit comfortably in place at the dog's neck. Repeated measurements on a single dog showed that the reproducibility of the position was satisfactory. With this vastly improved counting device a new interest was taken in thyroid measurement.

Uptake of I^{131} by the Thyroid Gland

To obtain information regarding the retention of iodide by the thyroid gland and the whole body, three dogs, 0499, 0500, and 0503, were set aside for ingestion of I^{131} and subsequent measurement and collection of urine and feces. They were kept in individual metabolism cages for several days

preceding the administration of iodine in order to acclimate themselves to the new environment. The I^{131} was administered orally in a gelatin capsule. Immediately after administration of the isotope the animals were fed their regular diet. In this way the iodine was in company with the normal dietary complement of iodide. Urine collections were begun at the time of feeding. Following ingestion of the isotope the animals were counted in the whole-body counting facility with the 4 in. x 8 in. crystal. They were placed on the cart, and thyroid measurements were made at 3, 6, 10, 12, 24, and 48 hours, and daily thereafter for a week. Whole-body measurements were made daily for the same period. The very rapid thyroid uptake of I^{131} is clearly discernable. The peak of uptake was reached at approximately two days in each of the animals. After this time the measurements were made with less regularity for the remainder of the 12 days. The maximum per cent uptake varied over quite a range from 8% to 15% at two days. The slopes of the subsequent loss of iodine from the thyroid were very similar for the several dogs. Whole body retention was similar in each of the three dogs. They were not measured for a long enough period of time to establish the break noted in the earlier animals although the retention at 10 days indicated a break from the previous slope.

Excretion of I^{131} in Urine and Feces

Urine collections were made at 12, 24, and 48 hours after administration of the I^{131} . Blood samples taken by jugular puncture were collected at 2, 6, 10, and 24 hours. Because of difficulties with the counting equipment many of the data from these collections were lost. Radioactive measurements of the urine, blood, and feces from dog 0503 were made and are presented in Table 9.

TABLE 9

I^{131} concentration in blood

Time, hr	Per cent of injected dose
2	9.9
6	11.2
10	10.6
24	8.6

Excretion of I^{131}

	Time, hr	Per cent of injected dose	Per cent of remaining I^{131}
Urine	12	19.4	19.4
	12-24	14.1	17.5
	24-48	28.0	42.1
Feces	24	0.5	0.5
Total	48	62.0	-

Although the I^{131} was administered orally to each dog with the daily diet, it can be noted from the table that by two hours after ingestion there was a substantial amount of the isotope already in the blood. The amount of I^{131} in the blood did not change significantly during the 24-hour period following ingestion of the isotope. There is the suggestion of a drop in blood iodide by 24 hours. It is regrettable that this was not followed up by samples of blood taken at later times. The excretion data clearly show that the bulk of the iodide is excreted in the urine with an insignificant amount in the fecal material. The rate of excretion remains relatively constant during the 48-hour period during which data are available. Even though the per cent of the ingested dose excreted during each of the time periods indicates that there is a decrease in excretion rate, this interpretation is misleading because of the relatively large loss of isotope during each collection period. The corrected value based upon the per cent of the dose remaining after each of the collection periods is much more revealing and indicates that the rate is not changing appreciably. This would be expected if iodide is treated nonselectively by the kidney or if there were no mechanism for its conservation. With no change in the physiology of the animal there would be less than 5% of the injected dose remaining in the body at 8 days, based upon the excretion figures alone. This is a complicated picture because of the uptake and subsequent release of iodide from the various organs in general, and the thyroid, stomach, and integument in particular. Each of these organs concentrates and releases iodide at different rates. To explore these relationships would require an extensive sacrifice series in which various organs and tissues could be sampled at varying times after ingestion of I^{131} . This is not practical at the present time.

Dietary Intake of Iodide and Release Rate from Thyroid

To test the effect of iodide consumption on subsequent uptake and release of I^{131} four dogs, 0174, 0272, 0275, and 0881, were put on four separate diets containing differing amounts of inorganic iodide. The animals had all been raised on the semisynthetic diet of Kaspar.⁽⁴⁾ The normal semisynthetic diet contains 165.7 μg of iodide per 100 g of food. This diet was given to dog 0174. His daily food consumption averaged 322 g, giving a daily intake of 534 μg of iodide. Dog 0281 was fed a diet containing 1/5 the normal amount of iodide. With an average food consumption of 195 g/day this provided 65 μg iodide per day. Dog 0275 consumed 265 g/day of a diet containing 1/50 the normal amount of iodide (3.3 $\mu\text{g}/100\text{ g}$) giving a daily total of 8.7 μg iodide. Dog 0272 had no iodide added to his diet and consumed 227 g per day. The iodide intake was virtually zero. The animals were maintained on these diets for 28 days prior to the ingestion of I^{131} . They were measured with the thyroid crystal and whole body counter for a period of 16 days. At the end of this period they were given a second capsule containing I^{131} and measured for radioactivity for another 18 days. During this entire period the animals were maintained on their various diets. The data from the thyroid and whole-body counts have been combined with those from all other dogs measured to date and are presented in Tables 10 and 11.

TABLE 10
Retention of ^{131}I by the body as a whole

Dog no.	Time elapsed, days	Biological retention, fraction of original dose	Dog no.	Time elapsed, days	Biological retention, fraction of original dose	Dog no.	Time elapsed, days	Biological retention, fraction of original dose
0078	0.0183	0.6399	0233	1.0175	0.2501	0281	13.7396	0.06873
0078	1.1829	0.3688	0233	2.0833	0.1239	0281	16.8125	0.06186
0078	2.2079	0.2244	0233	2.7993	0.09279	0281	17.7799	0.05540
0078	2.9302	0.1889	0233	3.8590	0.09126	0281	21.0250	0.05832
0078	3.9850	0.1468	0233	6.9868	0.07295	0499	0.0368	0.5216
0078	6.9940	0.09027	0233	7.8063	0.07137	0499	1.1125	0.5050
0078	8.1496	0.07404	0233	8.8590	0.06783	0499	2.0785	0.3239
0078	9.2142	0.06633	0233	9.7882	0.06454	0499	3.0250	0.1809
0078	9.9274	0.06141	0233	10.7542	0.06945	0499	8.0326	0.1079
0078	11.0149	0.06094	0233	13.7618	0.05980	0499	10.1792	0.1074
0078	14.1142	0.04186	0272	0.9403	0.3590	0500	0.0410	0.6725
0078	14.9550	0.05176	0272	2.9306	0.2926	0500	1.1306	0.5972
0174	0.9361	0.2475	0272	3.8292	0.2551	0500	2.0854	0.4707
0174	2.9250	0.1092	0272	6.7958	0.1838	0500	3.0361	0.2659
0174	3.8243	0.1319	0272	13.7375	0.1311	0500	8.0493	0.1024
0174	6.7917	0.1132	0272	16.7958	0.07443	0500	10.1889	0.08685
0174	13.7306	0.07513	0272	17.7764	0.08888	0503	0.0458	0.5949
0174	16.7910	0.08068	0272	21.0083	0.06791	0503	1.1389	0.4779
0174	17.7715	0.06442	0275	0.9493	0.3881	0503	2.0938	0.2750
0174	21.0007	0.06805	0275	2.9333	0.3045	0503	3.0458	0.1566
0181	0.0136	0.7540	0275	3.8389	0.2766	0503	8.0743	0.07069
0181	0.7351	0.6298	0275	6.8056	0.2380	0503	10.2000	0.05883
0181	1.1136	0.6039	0275	13.7396	0.1630	0570	1.0527	0.6315
0181	1.1198	0.6097	0275	16.8076	0.1367	0570	3.9221	0.2368
0181	1.7358	0.4338	0275	17.7792	0.1322	0570	7.0082	0.08879
0181	2.1198	0.3766	0275	21.0208	0.1120	0570	9.0742	0.06202
0181	4.0782	0.1088	0281	0.9569	0.2635	0570	10.9575	0.05054
0181	7.0754	0.05707	0281	2.9347	0.1613	0570	16.1895	0.04968
0233	0.0139	0.5084	0281	3.8431	0.1411	0570	21.1513	0.05645
0233	0.0972	0.5327	0281	6.8104	0.1162			

TABLE 11
Retention of ^{131}I by the thyroid

Dog no.	Time elapsed, days	Biological retention, fraction of original dose	Dog no.	Time elapsed, days	Biological retention, fraction of original dose	Dog no.	Time elapsed, days	Biological retention, fraction of original dose
0174	0.9403	0.07719	0272	16.7535	0.04118	0281	14.7882	0.03360
0174	1.0021	0.06623	0272	16.8882	0.05315	0281	15.7875	0.03635
0174	1.9326	0.08385	0272	17.9882	0.05245	0281	16.7639	0.03885
0174	1.9500	0.07864	0275	0.9451	0.1489	0281	16.8882	0.03233
0174	2.9625	0.07617	0275	1.0139	0.2425	0281	17.9886	0.03002
0174	2.9785	0.07001	0275	1.9368	0.2222	0499	0.1111	0.02826
0174	3.7778	0.07714	0275	1.9625	0.2653	0499	0.2340	0.04013
0174	3.9451	0.06652	0275	2.9736	0.2152	0499	0.4132	0.05431
0174	6.9500	0.06292	0275	2.9847	0.2221	0499	0.5000	0.05313
0174	7.9972	0.05607	0275	3.7826	0.1902	0499	1.2028	0.07764
0174	9.9618	0.05228	0275	3.9563	0.2235	0499	2.1069	0.08093
0174	11.0285	0.05199	0275	6.9632	0.1670	0499	3.0715	0.06294
0174	13.7861	0.05855	0275	8.0104	0.1166	0499	6.2840	0.03541
0174	13.7924	0.04707	0275	9.9750	0.1327	0499	8.0979	0.02638
0174	14.7861	0.05481	0275	11.0396	0.1084	0499	10.1007	0.02512
0174	15.7840	0.05841	0275	13.7910	0.1067	0500	0.1167	0.03537
0174	16.7493	0.04393	0275	13.8035	0.1032	0500	0.2410	0.05340
0174	16.8882	0.05546	0275	14.7896	0.1137	0500	0.4250	0.06345
0174	17.9847	0.04555	0275	15.7882	0.1182	0500	0.5056	0.07940
0272	0.9444	0.2005	0275	16.7611	0.09338	0500	1.2090	0.1286
0272	1.0063	0.1694	0275	16.8896	0.09361	0500	2.1125	0.1366
0272	1.9361	0.1878	0275	17.9896	0.09166	0500	3.0792	0.1355
0272	1.9542	0.2089	0281	0.9438	0.09432	0500	6.2903	0.09638
0272	2.9660	0.1825	0281	1.0167	0.08198	0500	8.1049	0.07454
0272	2.9819	0.1926	0281	1.9361	0.09298	0500	10.1097	0.05167
0272	3.7813	0.1682	0281	1.9653	0.1023	0503	0.1236	0.03482
0272	3.9493	0.1642	0281	2.9764	0.09367	0503	0.2479	0.05160
0272	6.9556	0.1012	0281	2.9840	0.1112	0503	0.4326	0.05510
0272	8.0014	0.1026	0281	3.9590	0.09073	0503	0.5125	0.08057
0272	9.9660	0.08424	0281	6.9688	0.06309	0503	1.2167	0.09853
0272	11.0319	0.04658	0281	8.0160	0.05722	0503	2.1194	0.1019
0272	13.7896	0.06717	0281	9.9778	0.05613	0503	3.0868	0.09604
0272	13.7958	0.04817	0281	11.0424	0.04913	0503	6.2972	0.05602
0272	14.7889	0.06826	0281	13.7896	0.03762	0503	8.1208	0.04991
0272	15.7875	0.06275	0281	13.8069	0.04415	0503	10.1201	0.04253

Thyroid and Whole Body Retention of I^{131} - Combined Data

While each of the procedures outlined above served some particular purpose, each yielded some information common to all methods. This was with regard to the loss of iodide as indicated by I^{131} from either the body as a whole or from the thyroid in particular. It is pertinent to point out that the data obtained from the analyzer are transformed into microcuries and then into fractional retention by a particular program for the IBM 1620 computer. The results obtained from the computer are in terms of both biological and physical properties and constitute effective retention. These are not such useful data for tracer studies of this type as for the assessment of radiation dosage due to the presence of absolute amounts of radioactive substances. It was desirable to know the biological retention and so I devised and wrote a simple program for the computer which took the data from the existing program and converted it to biological retention by correcting for the amount of physical decay at the time of measurement. The biological retention program was written in so that the output from it could be used as input for the least-squares program for the determination of the best-line fit of the data. In this way the data were submitted to further analysis and could be grouped in different ways to examine the slope of the biological decay.

The first point to be noted regarding all the data from the various animals is that the loss of I^{131} from the thyroid or from the body as a whole is neither a linear function nor is it a single exponential function. A simple plot of the data on semilog paper reveals the change in slope of the best-line fit as time after administration proceeds. By grouping the data in several ways and trying each with the least-squares program, it was apparent that the most consistent break in the slope was at about 8 days after administration of the isotope whether whole body or thyroid counts were used. On this assumption the data were arranged as in Table 12 and the

TABLE 12
Retention of I^{131}

Dog no.	Interval, days	Biological half-life, days		Dog no.	Interval, days	Biological half-life, days	
		Thyroid	Whole body			Thyroid	Whole body
0078	0-2	-	1.5	0375	2-8	6.5	11.5
	2-8	3.1	4.0		9-18	20.1	13.5
	9-15	-	12.3		0-18	-	11.9
0174	0-15	-	4.3	0281	2-18	11.6	-
	2-8	14.0	118		2-8	5.9	8.7
	9-18	72.2	39.6		9-18	10.0	29.0
	0-18	-	13.8		0-18	-	9.7
	2-18	23.5	-		2-18	9.0	-
0181	0-2	-	2.1	0499	0-2	-	3.0
	2-8	2.5	3.2		2-8	4.0	6.7
	0-8	-	1.7		0-8	-	4.2
0233	0-2	-	1.0	0500	0-2	-	4.0
	2-8	7.4	12.0		2-8	5.9	4.3
	9-14	-	28.9		0-8	-	3.2
	0-14	-	4.9		0-2	-	1.9
0272	2-8	5.3	5.9	0503	2-8	5.2	4.8
	9-18	19.4	8.1		0-8	-	3.0
	0-18	-	8.4		1-7	-	2.1
	2-18	7.5	-		9-21	-	173
				0570	0-21	-	6.2

half-time of each segment of the decay curve was calculated. There is an initial rapid loss of iodide, which takes place in the first two days, due to excretion. This may be noted in the whole-body retention curves. The break at 2-3 days is less dramatic than that at 8 days but is probably significant.

Examination of the half-periods for thyroid retention reveals that there is a greater degree of uniformity in the loss of I^{131} from them during the period from 2-8 days than there is at any later time. With the exception of dog No. 0174, the animals range from 4.0 to 6.5 days for the half-period in that segment between 2 and 8 days after administration of the isotope. There is less uniformity in the same period with regard to whole-body retention although in several animals the half-period for whole-body retention is very close to that of the thyroid. One would expect this because of the large share of the I^{131} which is found in the thyroid during this period. There is, however, no such nice correlation between whole-body and thyroidal half-periods in the times after 8 days. Although a number of interesting trends are to be noted in these findings they are without real significance because of the variety of conditions under which the various data were collected.

In particular animals 0174, 0272, 0275, and 0281 are to be set aside from the others because of the nature of the diet to which each was exposed for a prolonged period. It is my feeling that dog 0174, while acting as a control for the diet experiment, is not normal because of the deviation of all information gathered for him from that taken from the other animals, whether in this experiment or not. The uptake was depressed, and the retention was very high, indicating a very slow turnover of iodine. It is regrettable that more was not known about this at the time so that a better assessment of this dog and its condition might have been made. The uptake of iodine by the other three dogs was as might be expected where there was iodine deprivation. It is curious to note that dog 0275, which had only $8.7 \mu\text{g}$ of iodine per day, was higher in uptake and retention than dog 0272 which had no iodine added to its food. Both 0272 and 0275 exhibited greater retention of I^{131} than did either 0174 or 0281. It would appear that the dogs with the least iodine in their diet were conserving that which they had in a better fashion than were the ones with sufficient or at least greater iodine intake. That this conservation, as measured by the per cent retention, is not greater may be explained by the fact that the tracer amounted to a much larger fraction of the total daily iodine intake in those dogs deprived of the normal amount than it did in the normal animals.

The data have been turned over in several ways in an attempt to arrive at some meaningful comparative figures for the amount of iodine in the thyroid. Inasmuch as a supposed equilibrium existed at the time of measurement between the dietary intake and the secretion, it was felt that some approximation of thyroidal iodine content could be made. Considering that the maximum per cent uptake reflected the daily turnover of iodine,

and that the daily iodide intake was measured, and that the daily loss from the thyroid could be measured from the slope of the release, a ratio was set up whereby the maximum per cent uptake by the thyroid times the micrograms uptake divided by the per cent released per day would equal the total micrograms in the thyroid. The figures obtained for the several animals showed great variation but indicated that dogs 0275 and 0281 were deficient in thyroidal iodine. There are some serious errors in such rough calculations, but they may serve to indicate relationships not otherwise seen.

Protein-bound Iodine Determinations

A microanalytical kit for the determination of protein-bound iodine (PBI) was purchased from Hycel, Inc. This apparatus utilizes only 0.3 ml of serum for a determination. The technique involved in the determinations is very involved and includes precipitation, boiling, and later distillation of the iodide. All reagents were purchased with the kit in order to insure their purity.

Many samples of serum and standards have been run through the procedure. As a result it is possible to give a range of PBI for the normal animals of about 3-8 $\mu\text{g}/100$ ml serum. The inorganic iodide in the serum ranges from 0.1-5.0 μg . The technique has not been applied to many animals, however, because the range which is given above will be found not only between animals but in duplicate samples taken from one animal or from pooled serum from several animals. There has been a great deal of difficulty encountered in perfecting the technique to the extent that reproducibility is possible. The technique requires that time be available for continuation of sample processing once it has begun. Because of the lack of technical help this has been impossible when an experiment has been under way because of the demands of the counting procedures.

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DRY EMULSION AUTORADIOGRAPHY

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The resolution of an autoradiogram depends in part on the distance between the source of irradiation and photographic emulsion. Close approximation of emulsion to tissue is usually achieved by techniques utilizing flotation on water or liquid emulsion. A serious threat of translocation is thus introduced if the labeled substance investigated is water soluble. Dry application of emulsion to tissue with resolution at the cellular level is demonstrated.

The technique of autoradiography applied to tissues containing water-soluble substances has, in general, been unsuccessful because the methods of tissue processing and application of emulsion introduce a high probability of loss or translocation of the radioactively-labeled compound. A completely reliable method would be one in which all opportunities for translocation during the preparation of the tissue are eliminated. Described herein is a method for mechanically superimposing a nuclear emulsion upon an unwetted tissue section. To determine whether this technique would provide adequate resolution and reregistry at the cellular level, the method was tested with rat ileum sections from an animal injected with H^3 -thymidine. Such sections were used because they provide material in which the localization of the label in tissue is known.

Method

A glass cover slip $1 \times 2\frac{3}{8}$ in. was immersed in a gelatin-chromalum solution and dried. Pieces of autoradiographic stripping emulsion (Kodak Limited) 1×2 in. were floated on water using the technique described by Doniach and Pelc,(1) but with the emulsion side up. The film was then picked up on the prepared cover slip leaving a 1-cm long section at one end free of emulsion. The slips with emulsion were dried over calcium chloride in a light-tight box at room temperature.

The tissue section was prepared by standard fixation and paraffin imbedding techniques and was mounted on the etched side of a microscope slide centered about $\frac{3}{4}$ in. from the unetched end. The slide was dipped twice in xylol for 3 minutes to remove the paraffin and the xylol was removed by evaporation at room temperature.

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In a dark room with a Wratten No. 2 safe light, the free end of the cover slip was glued with an epoxy resin* to the etched end of the microscope slide carrying the tissue section. The "dry" emulsion and the tissue section were pressed together using a second shortened microscope slide ($1 \times 1\frac{3}{4}$ in.) which was placed over the cover slip and clamped with 6 paper clips** (three on each side). The short slide was used to avoid contact with the region of the epoxy bond. The assembly was then placed in a light-tight box containing desiccant calcium chloride, and allowed to stand in a refrigerator at 5°C.

After exposure for 2 weeks the clamps were removed, and the free end of the cover slip carrying the emulsion was separated slightly from the slide at the unglued end. An impermeable plastic bag was drawn over the tissue slide with separation maintained by insertion of a 2 mm rubber spacer at the end. The emulsion was processed at 20°C by immersing the assembly in Kodak D19 developer for 5 minutes, in water for 1 minute and in Kodak acid fixative for 8 minutes. The developed emulsion was allowed to dry at room temperature. The plastic bag was removed from the slide and the cover slip was similarly protected with a plastic bag. The tissue was stained with Celestine Blue (1 gm dissolved in 100 cc of water containing $\frac{1}{2}$ cc H_2SO_4 and filtered) for 5 minutes and then washed in distilled water, 70% alcohol, 95% alcohol, and finally absolute alcohol, each for 2 minutes. The plastic bag was removed from the cover slip and the assembly dried, first in room air and finally over calcium chloride, with the two portions separated by the 2 mm spacer at the unglued ends. The cover slip and microscope slide were reapposed carefully and sealed with Permount.

Results and Discussion

Current methods of cellular autoradiography utilizing liquid emulsions or stripping film techniques are unsuitable for work with water-soluble labeled compounds not firmly bound in tissue.(2,3) The mechanical application of film to tissue sections for autoradiography has been previously described.(4,5) However, these latter methods have not gained popularity, particularly in work requiring resolution at the cellular level because of difficulties encountered in reregistration. Epoxy resins provide a means of firmly bonding glass to glass and thus the danger of mismatching inherent in methods that use mechanical clamps or emulsions mounted on plastic is avoided.

Cells labeled with H^3 -thymidine provide a test system for evaluating resolution and reregistry. Figure 20 is an autoradiogram of rat ileum prepared by the method described herein. It is clear that the developed silver grains coincide closely with the established localization of H^3 -thymidine in the cell nuclei.

*Armstrong Adhesive C-4, Activator P. 4/1 mixture.

**L-E-B No. 2 Small, Cushman and Dennison Mfg. Co., New York, USA.

Figure 20. Autoradiogram of rat ileum prepared by the method described in the text

The problem of processing tissue by methods that avoid translocation of water-soluble substances prior to application of dry emulsion has not yet been solved. The demonstration that a useful degree of resolution at the cellular level can be obtained with "dry" emulsion methods is a first step toward providing a method for the autoradiographic study of water-soluble labeled substances at the cellular level.

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A RAPID BLOOD CATALASE SCREENING TECHNIQUE ADJUSTABLE TO ANY LEVEL OF ACTIVITY

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We wished to screen a large number of mice in order to detect a mutant exhibiting an abnormally low level of blood catalase. Since the definition of "abnormal" will depend on the normal level of blood catalase activity of a given strain of mice, it was necessary to devise a technique which could readily be adjusted to indicate "normal" or "subnormal" at any desired level of catalase activity. Using the procedure described herein, one individual can process perhaps 125 mice per day, including drawing of blood, preparation of solutions, and washing glassware.

The technique is based on perborate decomposition by catalase.⁽¹⁾ However, no titration is performed; after incubation of the blood with the substrate mixture, enzyme activity is halted by the addition of H_2SO_4 , and a small amount of KMnO_4 is added as an indicator to test for excess NaBO_3 . If the blood is low in catalase, residual perborate decolorizes the KMnO_4 . On the other hand, persistence of the KMnO_4 color indicates that sufficient catalase is present to decompose all perborate.

The screening test is adjusted to any desired level of blood catalase activity by varying the volume of blood assayed. This is accomplished by determining the length of blood collecting tube occupied by a known volume of fluid, then expressing volume in terms of length. Finally, the screening assay is standardized by means of a series of blood samples of known catalase activity.

Experimental and Results

Preparation of Buffered Substrate

A solution of sodium perborate was prepared by adding 2.5 g $\text{NaBO}_3 \cdot 4\text{H}_2\text{O}$ to 100 ml water and stirring with a magnetic mixer while adding concentrated HCl to pH 6.8. At this pH, solution is nearly complete, and the slight insoluble residue is removed by filtration. Nine volumes of this NaBO_3 solution mixed with one volume of $\text{M}/15$ phosphate buffer, pH 6.8, constitutes the substrate.

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Other Solutions

Other solutions necessary are:

- a) KMnO_4 , approximately 0.05 N. The exact concentration is not critical, since the KMnO_4 serves merely as an indicator of the presence or absence of excess NaBO_3 .
- b) 4 N H_2SO_4 containing 0.2% MnCl_2 .
- c) Octyl alcohol in a dropper bottle.

Procedure

The reaction is carried out in 50-ml Erlenmeyer flasks, to each of which 1 ml of distilled water is initially added. Blood is collected by the orbital sinus technique(2) in heparinized microcollecting tubes,* to the desired point which is previously marked on the tube. The blood is transferred to the water by blowing and rinsing a few times. The blood is probably completely laked within 30 sec or so, but it is convenient to set up 10 or 15 such flasks at a time, and there will be no possible question of incomplete laking. In front of each flask is placed a stop watch, and the mouse whose blood is being assayed is kept in a small individual cage behind the flask.

As the stop watch is started, 20 ml of buffered substrate is added to the flask. For this purpose, an automatic dispensing pipet is convenient. The mixture is briefly swirled to mix. After 10 min at room temperature, 5 ml of the H_2SO_4 - MnCl_2 solution is added by means of another dispensing pipet. This halts the enzyme activity and provides the acid milieu necessary for the NaBO_3 - KMnO_4 reaction. The MnCl_2 serves as catalyst for reduction of the KMnO_4 .

A drop of octyl alcohol is then added to destroy the accumulated foam, and finally 2 ml of KMnO_4 is added with a third dispensing pipet. The flask is mixed by swirling, and the color is noted. A blue color persisting for at least 15 or 30 sec indicates catalase activity above the level being tested; decoloration indicates a lower catalase activity.

Relation of Length to Volume of Blood Collecting Tube

Since our prime interest was maximal speed and efficiency within a satisfactory range of accuracy, we wished to omit such steps as transferring blood from collecting tube to measuring micropipet. In a preliminary

*Micro blood collecting tubes, Catalog B 3095-2, Scientific Products, Evanston, Illinois.

experiment we, therefore, measured the length of collecting tube occupied by 100 μ l of water. The mean for 9 different tubes, 3 from each of 3 separate packets, was 50.2 mm, with a standard deviation of 1.03 mm. We have, therefore, used the value of 1 mm length as equivalent to 2 μ l volume. A slight error is introduced by the constricted tip of the tube, and if one were testing for an extremely high blood catalase level (i.e., very small amounts of blood used), this might be an appreciable factor. However, in a screen for low catalase (i.e., large amount of blood used), this minor factor may be neglected.

Standardization of Screening Assay

A series of 15 normal mice was killed by cutting the throat, and the blood was caught on watch glasses on which a little heparin solution had been dried down. The blood was briefly mixed to dissolve the heparin, then transferred to 5-ml beakers containing micromagnetic stirrers and covered with small watch glasses. After gentle stirring, samples were nearly simultaneously removed by two individuals, one of whom performed a quantitative catalase assay,(1) and the other of whom performed the screening assay described above. In the latter case, catalase activity was tested at 30 mm and at 35 mm of blood in the collecting tubes. Depending on the results, the test was then repeated on a graded series of blood lengths spaced at 1-mm intervals. The blood length at which an intermediate color was obtained was considered the "equivalence length;" if no intermediate color was seen, the equivalence length was considered to be halfway between those of the adjacent positive and negative tubes. Table 13 lists for each mouse the "equivalence length," the catalase activity, and their product. Since this product is a constant, the mean product can be used to determine the length of blood sample necessary to screen for catalase at any desired blood level.

TABLE 13

Relationship of quantitative blood catalase activity to screening test "equivalence length"*

Mouse	Quantitative assay (PU/ml)**	Screening assay (equivalence length in mm)	Product
1	101	37.0	3740
2	108	36.0	3890
3	109	36.0	3920
4	111	37.5	4160
5	111	36.5	4050
6	112	35.5	3980
7	117	34.0	3980
8	119	33.0	3930
9	125	34.5	4310
10	127	36.5	4640
11	129	31.5	4060
12	133	30.5	4060
13	137	28.5	3900
14	142	32.0	4540
15	146	27.5	4020
Average:			4080 $\pm$ 250

*The "screening assay equivalence length" is that length of blood column which gives a result just intermediate between a "positive" and a "negative" test.

**Perborate units per ml.

Accuracy of Screening Assay

The accuracy of the screening procedure was tested on 30 mice having blood catalase levels ranging from 93 to 147 perborate units (PU) per ml. (These levels were not made known to the experimenter until the test was completed.) The screening value was set at 35 mm of blood (equivalent to 117 PU/ml). Twenty-eight of the 30 mice were properly classified by the screening test. One mouse, blood catalase 108 PU/ml, was erroneously called high-catalase (i.e., greater than 117 PU/ml), and one, blood catalase 126 PU/ml, was erroneously called low-catalase (i.e., less than 117 PU/ml).

No explanation is available for the latter of these errors, but the former is probably due to the delayed decoloration of KMnO_4 , which is occasionally seen even in the presence of excess reducing agent. This occurred in an early experiment, before MnCl_2 was routinely added as a catalyst of KMnO_4 reduction.

Discussion

This screening technique is not intended to serve as a substitute for a quantitative assay. It has, however, three particular advantages as a screening test: a) it is nondestructive, b) it is quite rapid, and c) it is readily adjustable to separate a population into groups with blood catalase higher than, and lower than, any desired level.

It is necessary to prepare the substrate with somewhat more care than in the quantitative perborate assay for catalase.⁽¹⁾ In the latter test, two flasks containing no catalase source are incubated simultaneously with each set of experimental flasks. These control flasks then serve as a measure of the perborate present before catalase action. However, there are no control flasks in the screening method described, hence the need for more careful preparation of the substrate.

The only solutions for which the concentration or volume is critical are the substrate solution and the added blood. H_2SO_4 and MnCl_2 are both present in considerable excess. The KMnO_4 serves merely as an indicator, and its exact concentration or volume becomes of importance only if the amount of blood added is nearly exactly that necessary to decompose the NaBO_3 present. The de-foamer octyl alcohol is innocuous if added after the reaction has been halted by the H_2SO_4 . In one experiment it was added about a minute before the H_2SO_4 , and the results seemed to show an inhibition of the catalatic activity.

The color must be observed fairly shortly after addition of the KMnO_4 , since almost all bloods will slowly decolorize a solution of KMnO_4 by a nonenzymatic reaction.

In the interests of time, we have tried to eliminate the preliminary laking of the blood in water. However, the buffered perborate which serves as substrate appears to be approximately isotonic, and we have found that unlaked erythrocytes exhibit slightly less than half the catalase activity of laked cells. In addition, the behavior of such an unlaked preparation appears to be somewhat more erratic than that of laked blood. We tried to incorporate into the buffered substrates such lysing agents as saponin, detergents, and deoxycholate. Of these, only the deoxycholate lysed the cells sufficiently rapidly, but so much deoxycholate was necessary that the solutions tended to gel and became very difficult to handle.

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EFFECT OF PUROMYCIN ON BLOOD AND LIVER CATALASE

Robert N. Feinstein, Judith B. Howard, and John E. Seaholm

Puromycin (aminonucleoside) has been reported to inhibit protein synthesis.⁽¹⁾ Since we have been attempting for a long time to develop techniques for reducing the catalase activity of blood and tissues, and for maintaining it at a low level for prolonged periods of time,⁽²⁾ it appeared worth while to see whether a general inhibitor of protein synthesis, such as puromycin, would also have long-term effects on the catalase level.

Experimental

Mice were CF No. 1/An1 males, about 6 weeks of age. Puromycin (Nutritional Biochemicals Corporation) was injected intraperitoneally at 100 mg/kg, twice daily. In the first experiment, the puromycin was injected on days 1, 2, 3, and 4, and blood and liver catalase assays were conducted on days 2, 3, and 5. In the second experiment, puromycin was injected twice on day 1 only, and animals were sacrificed and assayed on days 2, 3, and 5. Values for three mice were averaged for each point, and in each case control animals were injected with saline and assayed in the same manner as the puromycin-treated mice.

Catalase assays were performed by the perborate method,⁽³⁾ the liver being homogenized and diluted with 1% Triton X-100 for maximum expression of catalase activity.⁽⁴⁾

Results

Table 14 shows the effect of eight injections of puromycin on blood and liver catalase activity. It is evident that, although erythrocyte catalase

TABLE 14

Effect of repeated injections of puromycin on blood and liver catalase

Day	Liver catalase			Blood catalase		
	Saline	Puromycin	Difference, %	Saline	Puromycin	Difference, %
2	960	895	-7	165	140	-15
3	991	635	-36	153	165	+8
5	1003	441	-56	162	184	+14

Mice were injected intraperitoneally with 100 mg puromycin per kg, or with saline, on days 1, 2, 3, and 4. They were sacrificed and assayed in groups of three, except for the last puromycin datum, where only one survivor was available. Catalase activities are expressed as perborate units per gram of liver or per ml of whole blood.⁽³⁾

is unaffected, the level of catalase activity in the liver progressively decreases, until by the fifth day after beginning puromycin treatment, catalase activity is less than half that of control animals. By this time, animals have lost considerable weight, have a scruffy appearance, and are obviously in precarious physical condition. Actually, some deaths had occurred in the treated animals by the time of last assay.

Another set of mice was injected twice only, on day one, and groups of three were sacrificed at various times thereafter and assayed for liver catalase. (Blood catalase assays were not included, since even the more drastic treatment of the first experiment had shown no effects on blood catalase.) The results are shown in Table 15, which indicates a maximum of only 22% decline in liver catalase by day 5, and return to normal by day 8. In this treatment no drug toxicity was evident, and no animals died from the treatment.

TABLE 15

Effect of one-day injection of puromycin on liver catalase

Day	Liver catalase	
	Saline	Puromycin
2	854	910
3	870	850
5	910	710
8	not done	940
		Difference, %
		+7
		-2
		-22
		+7*

*Based on average of other controls.

Mice were injected intraperitoneally twice on day one only, with puromycin, 100 mg/kg, or with saline. They were sacrificed and assayed in groups of three. Catalase activities are expressed as perborate units per gram of liver.⁽³⁾

Discussion

These experiments are obviously only preliminary, designed to learn something of the toxicity of puromycin, and its possible use in long-range inhibition of protein synthesis. Thus far the experiments suggest a conclusion that might have been predicted, namely that if protein synthesis is significantly affected for a prolonged time, the effect on the animal is likely to be drastic. Further, since it is a simple matter to reduce the catalase activity of the liver but not of the erythrocyte,⁽⁵⁾ our prime interest lies in effects on the erythrocyte, and here we find that relatively short-term

experiments (e.g., 8 days) are inadequate to demonstrate catalase diminution in the red cell. Again this was a predictable finding, since inhibition of protein synthesis would not be expected to affect protein of circulating erythrocytes.

Future experiments will be oriented toward an attempt to inhibit catalase synthesis in the bone marrow long enough to permit the later output of a wave of catalase-free erythrocytes.

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LACK OF EFFECT OF EXOGENOUS CATALASE ADMINISTERED DURING PREGNANCY ON CATALASE CONTENT OF PROGENY

Robert N. Feinstein, John E. Seaholm, and Judith B. Howard

We have been interested in techniques for obtaining animals low in total body catalase.⁽¹⁾ A suggestion was made* that perhaps the introduction of significant amounts of exogenous catalase during pregnancy might produce litters of animals of low body catalase. We have now tested this suggestion and have found that the added catalase is without effect on the liver or blood catalase of the progeny.

Materials and Methods

Mice were CF No. 1 males and females, approximately 8 weeks of age. Each of the 5 males was housed with 6 females. In each such group, 3 females were marked by ear clip to receive catalase, and 3 were marked to receive saline. Counting from the day of housing the animals together, females received catalase (or saline) on days 3, 5, 7, 10, 12, 14, and 17. For final assays, matched litters (one catalase, one saline) were selected which were characterized by (a) having the same sire, and (b) having as nearly as possible the same birth date. Mortality was heavy, and it was finally possible to use only two matched pairs, totalling 10 catalase-treated mice and 11 controls.

Catalase used was Caperase,** a stable, dry beef-liver catalase which was readily reconstituted by the addition of saline. The amount of each intraperitoneal injection was sufficient to add 35% to the total body catalase of the recipient, as determined by whole body catalase assays of other mice of the same original shipment.

Blood was drawn from the orbital sinus,⁽²⁾ and the animal was then killed by cervical dislocation. The liver was removed and frozen in a dry ice-acetone bath and was maintained on dry ice until assay, which was done in 1% Triton X-100 for maximum expression of catalase activity.⁽³⁾ All catalase assays were performed by the perborate method.⁽⁴⁾

Results

Results are presented in Table 16, which shows no significant difference in blood or liver catalase activity between the mice whose dam received catalase during pregnancy and those whose dam received saline.

*We wish to thank Dr. P. K. Lala for this interesting suggestion.

**We wish to thank Dr. P. Puig Muset, Laboratorio PEVYA, Barcelona, Spain, for generous gifts of Caperase.

TABLE 16

Blood and liver catalase activity of progeny of female mice receiving repeated catalase or saline injections during pregnancy

	Treatment of dams		
	Saline	Catalase	% Difference
Number of mice assayed	11	10	-
Average liver catalase, PU/g*	990	958	-10
Average blood catalase, PU/ml*	186	176	-9

*Perborate units per gram liver or per ml whole blood. (4)

Discussion and Conclusions

We have shown earlier(5) that intraperitoneally injected catalase rapidly appears in the blood stream but not in the liver. We have no information as to whether or not the catalase molecule can pass the placenta, or whether or not a high catalase level in the fetal circulation will diminish the synthesis of new catalase. The present data indicate only that repeated injections of exogenous catalase, presumably giving rise to repeated high maternal blood levels, is without ultimate effect on the catalase level in blood or liver of progeny.

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SEARCH FOR AN ACATALASEMIC MOUSE

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and Judith B. Howard

If a catalase-free strain of animal were available, it would provide a much needed research tool with which to test certain theories of radiation effects and oncology. The human studies of Takahara⁽¹⁾ and later of Aebi⁽²⁾ demonstrated clearly that it is possible for an acatalasemic mammalian organism to be perfectly viable, and so a project was conceived and begun here to search for a mouse genetically aberrant with respect to catalase activity.

Although Aebi⁽²⁾ screened his human cases with a simple all-or-none technique, it was decided here to determine the normal level and range of blood catalase, and then screen mice with a semiquantitative catalase assay which would distinguish normal from subnormal levels. Such an assay is described elsewhere in this report.⁽³⁾ This device, though entailing considerably more effort than Aebi's all-or-none screening, has the basic advantage that it will detect heterozygotes, whereas the other technique will screen out only homozygotes, a much more rare condition. In addition, it was decided to use not a normal mouse population, but a population consisting of progeny of irradiated sires. This should have the advantage of very considerably enhancing the possibility of finding a heterozygote, although one would not expect a homozygote to be produced as a result of parental irradiation.

Although we had first planned to do the irradiations here ourselves, it was pointed out to us that Dr. William L. Russell, Oak Ridge National Laboratory, was engaged in a massive program of irradiating sires and examining progeny for certain specific visible mutations. After such examination, these animals are ordinarily discarded. Dr. Russell very kindly consented to save these animals and send them on to us for biochemical testing for level of blood catalase. The sires of the mice tested received a total of 600 r of X-rays to the lower third of the body, in a divided dose of 500 r, then 100 r, or 100 r, then 500 r, spaced at 24 hour intervals.

One hundred and ten normal mice, exactly comparable to the mice to be screened except that the sires had not been irradiated, were assayed quantitatively⁽⁴⁾ for blood catalase. The mean and standard deviation from the mean were 149 ± 14 perborate units (PU) per ml whole blood. The range of 113-185 PU/ml will encompass 99% of the normal animals. The screening test level for subnormality was therefore set at approximately 110 PU/ml.

As of the end of 1963, approximately 5600 mice have been examined. From these, the screening test separated 19 mice as possible low catalase animals. All 19 were quantitatively assayed, and five were found to have blood catalase of above 120 and so were discarded. The remaining 14 may be grouped as follows:

111-120 PU/ml	3
101-110	6
91-100	4
below 91	1.

The mouse listed as "below 91" is a female whose actual blood catalase is 40 PU/ml. She is undoubtedly a heterozygote. Considerable genetic data will have to be accumulated before it can be determined whether the other animals listed above are actual mutants or merely "low normals."

The female of 40 PU/ml was pregnant when received. She has since produced a litter of four males, the blood catalase activity of which is 75, 141, 150, and 123 PU/ml. It is hoped that the backcross of the mother by her low catalase son will produce a homozygote.

We wish to emphasize one particular point. Not only do we feel fairly confident that we will eventually obtain a strain of acatalasemic mice, but we feel that we have demonstrated the feasibility of obtaining, by comparable techniques, other mutants suitable for tools in the study of various inborn errors of metabolism. The requirements are (a) a supply of progeny of irradiated parents, and (b) a rapid assay capable merely of distinguishing between normal and abnormal levels of some blood constituent. This need not be an enzyme. In fact, such disorders as phenylketonuria display their enzyme defects in the liver. In the particular case of phenylketonuria, however, it seems probable from the work of Hsia(5) that if the mice were simply kept on diets high in phenylalanine, the heterozygote would show a high blood phenylalanine. Other disorders, e.g., galactosemia(6) evidently represent erythrocyte defects, and a straightforward whole blood screen for the enzyme should serve to detect the heterozygote.

Summary

By means of a rapid, semiquantitative assay, approximately 5600 mice have thus far been examined for low blood catalase. Of these, 14 have been set aside as subnormal in blood catalase activity. Of the 14, one is almost surely a heterozygote, and some of the others may be. The feasibility of this approach in obtaining specific mutants for the study of inborn errors of metabolism is discussed.

We wish to acknowledge, with gratitude, helpful discussions with Drs. Herman Slatis (now at Michigan State University) and Max R. Zelle, Argonne National Laboratory; Drs. John B. Storer and Thomas Roderick of Jackson Memorial Laboratory; and Dr. William L. Russell, Oak Ridge National Laboratory. To Dr. Russell goes also our sincere thanks for his continuing cordial cooperation in providing us with the mice used in this study.

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ATTEMPTS AT RADIATION PROTECTION BY COMPOUNDS RELATED TO 3-AMINO-1,2,4-TRIAZOLE

Robert N. Feinstein and John E. Seaholm

We reported earlier that 3-amino-1,2,4-triazole (AT) had a small but significant protective effect when administered to mice before whole body X-irradiation.⁽¹⁾ We have recently been so fortunate as to have placed at our disposal a wide variety of chemical agents related to AT.* Since many of these are unusual compounds, not available elsewhere, it seemed desirable to make maximum use of them, and one facet of interest was their possible radioprotective ability. Some of these agents are characterized by the chemical groupings generally associated with many radioprotective agents, namely, a free mercapto group in proximity to an amino group.

Of the many compounds available (for complete list, see Reference 2), we selected several agents more or less representative of specific chemical groups. To a certain extent, also, our choice was dictated by the amount of compound still available after our primary testing had been completed.⁽²⁾

Experimental

Mice used were CF No. 1 females, about three months of age. X-irradiation delivered was 700 r to the whole body, at a rate of 36-37 r per min. X-ray factors were: 250 KV, 15 ma, 0.5 mm copper and 3 mm Bachelite filtration. The half-value layer was 1.72 mm of copper.

Drugs were administered intraperitoneally within ten minutes before the start of irradiation. Dosages are listed with the individual experiments. In some cases, because of limited solubility, it was necessary to inject the agent as a suspension in 5% gum arabic.

Results

Results are shown in Table 17. It will be seen that of the seven agents tested, six permitted greater survival, and slightly greater mean survival time, than their respective controls. The exception, 2-aminothiazole, appeared to be detrimental in that the mean survival time was very greatly shortened. In this latter group, first deaths did not occur earlier than in other groups; rather, all animals succumbed within an unusually sharply circumscribed period.

*We wish to express our warmest thanks to Dr. Cameron Ainsworth, of Eli Lilly and Co. Research Laboratories, for his cordial cooperation in supplying us with these compounds.

TABLE 17

Radioprotective ability of certain compounds related to aminotriazole

Expt.	Compound tested	Dose, mg/kg	No. mice	No. surviving	% survival	Mean survival time, days
1	Saline		16	0	0	12.7
	4-amino-3-mercapto- 1,2,4-triazole	500	16	3	19	14.0
	2-aminothiazole	250*	16	0	0	7.4
	5-mercapto-1,2,3,4-tetrazole	1000*	16	5	31	12.8
2	Saline		16	4	25	13.3
	1-methyl-5-mercapto- 1,2,4-triazole	150	16	9	56	13.4
	2-methyl-1,3,4,4H-oxadiazoline- 5-thione	600	10	4	40	14.7
3	Saline		16	1	6	12.3
	1-isopropyl-5-mercapto- 1,2,3,4-tetrazole	1000*	9	4	44	15.0
	1-tert-butyl-5-mercapto- 1,2,3,4-tetrazole	500*	11	2	18	14.1

*Indicates suspension in 5% gum arabic.

Discussion

These compounds were originally tested as inhibitors of catalase in vivo and in vitro.⁽²⁾ Since its catalase inhibitory power was the original reason for testing the radioprotective ability of AT, ⁽¹⁾ it is of interest to compare the catalase inhibitory property of these agents⁽²⁾ with the present tests of radioprotection. The results indicate clearly that there is no direct correlation. Thus, for example, 5-mercapto-1,2,3,4-tetrazole is the most effective catalase inhibitor yet developed, and the 1-methyl, 1-isopropyl, and 1-tert-butyl analogs thereof are totally devoid of catalase inhibitory ability, yet there is little distinction between them, so far as radioprotection is concerned. Although the radioprotective ability of these agents may still be in some way related to the enzyme catalase, the relationship is not immediately apparent.

Work on the radioprotective ability of these agents has been discontinued for several reasons: (a) unavailability of further supplies of some of the agents; (b) low level of radioprotection obtained; and (c) our understanding that some of these compounds have also been submitted to the master screening program for radiation protectors at Walter Reed Army Hospital.

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OCCASIONAL RESISTANCE OF MICE TO GLUCOSE OXIDASE INJECTION

Robert N. Feinstein and John E. Seaholm

In a recent publication,⁽¹⁾ we reported the drastic effects of intraperitoneal injection into mice of the enzyme glucose oxidase, a mold-derived product catalyzing the reaction: glucose + oxygen $\rightarrow$ hydrogen peroxide + gluconic acid. These effects, which depend qualitatively and quantitatively upon the dose administered, include erythrocyte hemolysis, methemoglobin formation, striking changes in hematocrit, and death.

During the course of that work it was observed that occasional animals - perhaps five per cent, although exact records were not kept - appeared to be totally unaffected by the enzyme. Injection of as much as 100 lethal doses caused no blood changes or other signs of toxicity in these mice.

We decided to investigate this unusual phenomenon, and we considered two possible explanations as most probable: (a) some sort of immune reaction,* or (b) a genetic phenomenon. In brief summary, neither of these possible mechanisms has provided a satisfactory explanation.

Experimental and Results

Immunological Testing

Three sorts of immunological testing were performed. In the first, we attempted to find antibodies to glucose oxidase in the plasma of mice known to have received, and survived, lethal doses of the enzyme, and in the plasma of normal mice. Blood was withdrawn from all mice by the orbital sinus technique,⁽²⁾ and the plasma was separated by centrifugation. The plasma was placed in small tubes, and solutions of glucose oxidase of varying concentration (1.0-0.01 mg/ml) were carefully layered over the plasma. The interface was examined at intervals for signs of an antigen-antibody precipitate. The results of this experiment were negative. Although slight turbidities were occasionally seen at the interface, these were noted in both "resistant" and "normal" plasmas, and at no time was a definite precipitate observed.

The second immunological experiment was an attempt to immunize normal mice against glucose oxidase, to see whether their response to a later challenging dose of glucose oxidase resembled the response of the

*We wish to thank Dr. Bernard Jaroslow, Argonne National Laboratory, for his help in the immunological investigations.

occasional truly resistant mouse. For this purpose, 10 mice (CF No. 1 females, 10 weeks of age) were injected subcutaneously 6 times in the course of 12 days, each time with approximately $\frac{1}{3}$ of a lethal dose of glucose oxidase mixed with an equal volume of Freund's adjuvant. Nineteen days after the last immunizing dose, these 10 mice, plus 10 similar but untreated mice, were challenged with an intraperitoneal dose of approximately 4 lethal doses of glucose oxidase. All 10 of the untreated mice were dead within 6 hr, whereas only 3 of the treated mice were; the other 6 treated mice, however, appeared to be quite ill, and, in fact, 3 more died within the next 20 hr. It thus appears that a partial immunization was achieved, but it is considered that this bears little resemblance to the "resistance" phenomenon being investigated, for reasons discussed later.

The third test of the glucose oxidase resistance as an immune phenomenon was an attempt to transfer "passive resistance" from inherently resistant (4 mice), partially immunized (4 mice, as above), or normal mice (8 mice) to other presumed normal mice. This was done by mixing 200 microliters of the plasma in question with 50 μ l of glucose oxidase solution, and injecting the entire 250 μ l into the tail vein of a normal recipient. The dose of glucose oxidase was such that each animal received slightly more than one lethal dose. As a result of these injections, only one death occurred, and that was in the group receiving glucose oxidase plus plasma from a "partially immunized" mouse.

Genetic Testing

Because of the inconclusive, but generally negative, results of the immunological testing, and because of the possibility that a genetic sub-strain of CF No. 1 mice is resistant to glucose oxidase, we investigated the question from this point of view also. The following mice were challenged with 5 lethal doses of glucose oxidase:

- (a) Ten representatives of each of 11 genetically inbred strains;
- (b) Ten normal, previously untreated CF No. 1 mice; and
- (c) Eighteen CF No. 1 mice, the offspring of parents both of whom had proven resistant to injections of at least 20 lethal doses.

From results, given in Table 18, two general conclusions can be drawn:

- (a) None of the inbred strains is particularly resistant to glucose oxidase; and
- (b) The progeny of two resistant mice are not themselves resistant.

TABLE 18

Glucose oxidase challenge of various mouse strains and groups

	Sex	Age, weeks	No. challenged	No. surviving
"Resistant"*	M	7-10	10	0
"Resistant"*	F	7-10	8	1
CF No. 1	M	15-17	10	0
MA/J/An1	M	7-10	4	1
MA/J/An1	F	7	6	0
C57Br	F	ca. 35	10	0
RF	F	ca. 35	10	0
SWR	F	ca. 35	10	0
C57B1/6	F	ca. 20	10	2
LP/J	F	ca. 20	10	0
SJL	F	ca. 20	10	1
PL/J	F	ca. 16	10	1
129/J	F	ca. 20	10	0
A/d	F	ca. 20-24	10	0
BALB	F	ca. 20	10	1
CBA	F	ca. 20	10	0

*"Resistant" mice are first generation progeny of CF No. 1 parents, both of whom had survived challenge with about 20 lethal doses of glucose oxidase. Challenge in the present experiment was with 5 lethal doses.

Discussion

It appears that no explanation has yet been found for the phenomenon of erratic resistance to glucose oxidase. The genetic explanation seems ruled out almost entirely, since the progeny of two resistant mice are not themselves resistant. A slight remaining possibility is that the parents showed resistance because of genetic variance at different loci, hence the progeny will still not be homozygous for this trait. We intend to test this possibility when, and if, the proper mice become available, namely, a parent and offspring of the opposite sex, to permit backcrossing. At present we do not have enough survivors for this test.

At the same time, the immunological explanation also seems inadequate, for the following reasons, based largely on the second of the immunological experiments described above:

(a) Untreated mice give an all-or-none response to glucose oxidase; animals resistant to a given dose of glucose oxidase generally show

few or no toxic symptoms. The "immunized" mice in the present experiments were all in extremely poor physical condition, even though some eventually survived.

(b) The survival curve for normal mice given glucose oxidase is usually sharp and closely demarcated.⁽¹⁾ The "immunized" mice succumbed over a range of 12 or more hr.

(c) Since the 6-injection series produced only partial immunization, it seems extremely unlikely that a single mouse, in a cageful, would occasionally develop full-fledged immunity.

(d) The negative results of the first and third of the immunological experiments further bolster the conclusion that resistance to glucose oxidase is not an immunological phenomenon.

Summary

The occasional striking resistance to injection of glucose oxidase seen in apparently normal mice cannot be explained either as an immunological or as a genetic phenomenon.

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MUTATION INDUCED WITH CAFFEINE, ULTRAVIOLET LIGHT, OR 2-AMINOPURINE

Herbert E. Kubitschek and
Harold E. Bendigkeit

The dependence of mutation rate upon growth rate, that is, the mutational response, has been determined for several kinds of mutagen in strains of *Escherichia coli* B in continuous (chemostat) cultures limited with a variety of growth factors in minimal salts media. Both the magnitude of mutation rate and its dependence upon growth rate depend upon the factor used to limit growth when caffeine is the mutagen. When tryptophan-requiring cultures were limited with tryptophan, mutation rates did not depend upon growth rate but were constant. When cultures were limited with glucose, lactate, succinate, or phosphorus, mutation rates were directly proportional to growth rate. Parallel responses were observed for spontaneous mutation. Only one exception was found to these two kinds of response; a more complex dependence was found for nitrogen-limited cultures. It is thought that this complexity might be due to selection, since two different strains gave different responses when nitrogen was used to limit growth. With all other growth factors, mutation rates were the same for different strains. For this reason, it is thought that there are basically only two kinds of mutational response, and that in nitrogen-limited cultures the mutational response is modified by selection or by some other kind of secondary process.

The most direct interpretation of the two kinds of response is that the mutagen affects the gene either during or after its synthesis. If the mutagen acts only during the synthesis of DNA, then the mutation rate should be proportional to the replication rate, and hence to growth rate, as predicted by the copy-error hypothesis. Alternatively, if mutation occurs after synthesis then mutation should occur at an essentially constant rate, since DNA is present in relatively constant amount. This interpretation is supported by the responses with two other mutagens, ultraviolet light and 2-aminopurine. There is evidence that the first of these, ultraviolet light, acts upon DNA rather than its precursors. If so, mutation rate should be independent of growth rate for cultures limited either with glucose or with tryptophan. This constancy is found. For the second, 2-aminopurine, mutation is expected to result from the incorporation of this base analog into DNA. Hence mutation rate is expected to be proportional to growth rate for either limiting factor. This proportionality is also observed.

According to this interpretation, then, the observation of two different caffeine-induced mutational responses that depend upon the factor used to limit growth is a result of the occurrence of different processes

of mutation: The constant mutation rate observed with tryptophan-limited cultures is due to mutation occurring after synthesis of a gene, and the proportionality to growth rate for glucose-limited cultures is due to mutation occurring during synthesis. The nature of the changes in cellular processes leading to these different mutational responses is still unknown.

Since there has been no evidence that caffeine reacts chemically with DNA, we have been especially concerned about an explanation for the manner in which caffeine affects mutation rates in tryptophan-limited cultures. A possible explanation for this effect of caffeine comes from the observation of Ts'o(1) that caffeine reduces the thermal denaturation temperature of DNA. This observation leads to the following "localized melting" model: the presence of caffeine increases the spontaneous rate of mutation by increasing the frequency of spontaneous dissociation of duplex DNA along localized areas of the molecule. Both caffeine-induced and spontaneous mutation in tryptophan-limited cultures are thought to occur primarily in single separated strands of DNA. The frequency of separation increases when caffeine is present because the denaturation temperature is then lowered. A very similar alternative model that cannot be ruled out is that caffeine simply adsorbs to single strands as they are formed and prevents reformation of the duplex molecule.

The experimental support for this kind of model comes from an experiment with the dye acridine orange.(2) Although acridine orange was found to be an excellent photodynamic mutagen, the observation that the presence of acridine orange raised the melting temperature of DNA(3) led to the prediction that this dye would be a good antimutagen to caffeine in the absence of light. This result was found.

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COLLOIDAL PROPERTIES OF PLUTONIUM IN DILUTE AQUEOUS SOLUTION

Arthur Lindenbaum and William M. Westfall

The colloidal properties of Pu(IV) in solutions suitable for injection have been investigated as a function of pH, time after pH adjustment, and relative concentrations of Pu and complexing citrate ions. Techniques have been described for the preparation of Pu(IV) citrate solutions of minimal colloidal and for rapid estimation of colloidal formation by an ultrafiltration procedure. (1) Polymerization is increased with increasing pH and depressed by a large molar excess of citrate. Differences in the rates of polymerization and depolymerization are demonstrated. The rate of depolymerization is directly proportional to citrate concentration, time, and acidity. The polydisperse nature of Pu(IV) colloids is shown by kinetic dialysis studies suggesting the presence of several diffusible species. Apparent discrepancies between values for the fraction of diffusible Pu obtained by the ultrafiltration and dialysis methods are related to reversible polymerization.

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PROGRESS REPORT: PLUTONIUM REMOVAL. X. DISTRIBUTION AND REMOVAL OF MONOMERIC AND POLYMERIC PLUTONIUM IN THE RAT

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

The distribution and response to chelation therapy of both monomeric and polymeric forms of plutonium in the rat have been determined so that comparison may be made with earlier experiments in the mouse.^(1,2) The results summarized here will be published in full elsewhere.⁽³⁾ The distribution of the two forms of plutonium in rat tissues 3 days after intravenous injection again demonstrates that the partitioning of a hydrolyzable radioelement depends on its degree of polymerization. The polymeric form is deposited more heavily in the liver than is the monomeric, and the polymeric form is always deposited to a greater extent in liver than in bone. The major difference between the rat and the mouse is that the skeletal uptake of both forms of plutonium is twice as high in the rat as in the mouse - 12% versus 6% of injected polymeric plutonium and about 60% versus 24% of the injected monomeric form. The overall result is that the initial total-body burden is higher in the rat, since the deposition in liver is about the same for the two animal species ($3/4$ of the injected polymeric and about $1/3$ of the monomeric form).

The retention and loss of initial plutonium burdens in the tissues of rats in general followed the same patterns described in mice over the first 12 days: no change in bone burdens, relatively rapid loss of about $2/3$ of the deposited monomeric plutonium from the liver, and little if any loss of polymeric plutonium from liver. Over a 90-day period the rat liver lost $1/2$, and the mouse liver only $1/4$ of the deposited polymeric plutonium.

Therapy with the chelating agent diethylenetriaminepentaacetic acid (DTPA) (300 mg/kg injected intraperitoneally once daily from day 3 through day 11) in rats injected with either monomeric or polymeric plutonium had an effect similar to that found after DTPA therapy in mice. About $1/2$ of both forms of plutonium deposited in rat bone and almost all of the monomeric plutonium deposited in liver were removed. However, the therapy did not remove any polymeric plutonium from the rat liver, whereas it had removed about $1/3$ of that deposited in the mouse liver.

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PROGRESS REPORT: PLUTONIUM REMOVAL, XI. REMOVAL
OF POLYMERIC PLUTONIUM FROM MICE BY COMBINED
THERAPY WITH THE CALCIUM CHELATE AND
PENTAETHYL ESTER OF DTPA

Joan F. Markley

Diethylenetriaminepentaacetic acid (DTPA), one of the most effective agents for removal of plutonium deposited in the body, penetrates cellular membranes to a very limited extent; its action is essentially limited to extracellular plutonium. A certain proportion of plutonium in the tissues is intracellular, and this proportion is higher after administration of a colloidal form of plutonium. Thus, a change in the chelating agent to increase its cellular permeability is of interest. Since an ester form of DTPA would be expected to be more lipid soluble and, therefore, better able to penetrate cell membranes, the pentaethyl ester of DTPA was prepared by Dr. Norman Frigerio of this Division. This report summarizes the results of an experiment, published in detail elsewhere, (1) to compare the effectiveness of the pentaethyl ester and the calcium salt of DTPA in the removal of a polymeric form of plutonium from mice.

Daily intraperitoneal injections for 11 days of 75 mg/kg of the ester, either alone or immediately preceded by Ca-DTPA at 300 mg/kg, or of 300 mg/kg Ca-DTPA alone, were initiated in groups of 6 mice 3 days after intravenous injection of 0.092 μ c Pu²³⁹ in a highly polymerized form (16% ultrafilterable). The mice were killed either 3 days or 14 days after plutonium injection. Livers, spleens, and both femurs were removed for plutonium analysis.

The distribution of plutonium at three days, when treatment was begun, was typical of that obtained after injection of a polymeric solution: deposition was high in liver and spleen and low in the skeleton (Figure 21). Treatment with either Ca-DTPA or the DTPA ester given alone reduced the plutonium content of the liver by about 28% in each case. Combined treatment with both compounds gave a total reduction of 59%, which is the sum of either alone. The plutonium content of the spleen was increased by treatment with either agent alone, indicating redeposition of some of the plutonium removed from the liver. However, when combined treatment was given, this increase was prevented and the plutonium content of the spleen was reduced 60% below the level of the controls. The plutonium content of bone was reduced by treatment with Ca-DTPA alone, but was unaffected by the DTPA ester, and the effect of combined treatment on bone was the same as that of Ca-DTPA alone (Figure 21). Since the action of DTPA is essentially extracellular it is reasonable to assume that the additive effect of the DTPA ester on the liver is due to removal of intracellular plutonium. Autoradiographic studies should be of help in confirming this point.

Figure 21. The effect of treatment with Ca-DTPA and DTPA ester, singly and in combination, on the distribution of polymeric plutonium. Treatment was given once a day, beginning at 3 days after the injection of plutonium and continuing for 11 days. All values are the means from three pairs of mice. Bone content is obtained by multiplying the mean femur content by 20.

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RADIORESTORATION OF AMOEBAE AFTER INJECTION OF UNIRRADIATED PROTOPLASM

Edward W. Daniels and Evelyn P. Breyer

Supraethally X-irradiated (30 kr) Pelomyxa illinoisensis amoebae die within a week after exposure, but death can be prevented or delayed in 85 to 100% of the amoebae by transfer of unirradiated protoplasm (cytoplasm + nuclei) or cytoplasm alone, by direct microfusion. We have made many unsuccessful attempts to prevent death in these amoebae by micropipette injection of homogenates of unirradiated amoebae at 1-4°C. To separate the causes of failure of this procedure, we attempted to transfer unirradiated protoplasm into irradiated amoebae by micropipette rather than microfusion at 1-4°C. Thirty-six supraethally irradiated amoebae were injected with unirradiated protoplasm, isolated, and cultured, together with 47 untreated irradiated controls. Seven of the 36 treated amoebae reproduced to form mass cultures that had an average of 33 cells per clone within a month. There were no divisions among the irradiated controls. The remaining amoebae in the treated group lived significantly longer than their controls (16.0 ± 2.4 days versus 4.6 ± 0.14 days; $T = 6.03$).

THE METABOLISM OF MITOCHONDRIAL PROTEINS, I. THE ISOZYMES OF GLUTAMIC-PYRUVIC TRANSAMINASE

Robert W. Swick, Paula L. Barnstein,* and John L. Stange

We have been attempting to measure in an unequivocal fashion the metabolic turnover of various protein constituents of the liver; interest recently has focused on the mitochondrial proteins. Elucidation of the pattern of replacement of the individual enzymes and structural membranes may lead to a better understanding of the normal dynamics of this particle, its formation or assembly, "life-span," and disappearance. The first experiments, using isotopes, showed similar turnover rates for the purified structural protein and several crude fractions of the remaining proteins. This has suggested that the mitochondrion may be replaced as a unit.

The array of enzymes in this particle has usually been regarded as quite constant; e.g., cytochrome oxidase is frequently used as an index of the mitochondrial population. On the other hand, the activities of a number of enzymes in the cell have been shown to be subject to marked alteration as a result of dietary or hormonal manipulation. Most of the studies have been concerned with changes in activity in the whole cell, as measured in a homogenate, although some of these enzymes occur as isozymes, each of which may be found only in certain specific locations within the cell. A consideration of the effects of various treatments on the individual isozymes would be of significance. Also, if the activity of a mitochondrial isozyme is modifiable by treatment of the host, then the rate of change in its activity should be similar to the rate of synthesis of new mitochondria as measured by the incorporation of isotope into its protein. This would give an independent measure of the turnover rate of this structure.

This report is concerned with establishment of the existence of a mitochondrial isozyme of glutamic-pyruvic transaminase (GPT), with modification of its activity by various treatments, and with some chemical characteristics that distinguish it from the soluble isozyme.

Methods

Young adult female rats were fed diets of varying protein composition for periods of 5-10 days, or were made diabetic by injection with alloxan, or were injected with gluconeogenic corticosteroids (prednisolone and cortisone) for 2-4 days. Control rats were maintained on a 12% casein diet, as were all others except those on special protein diets. The animals

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were sacrificed by decapitation, and the liver was quickly removed and chilled to 0°. A 2% homogenate of the liver was prepared in water for estimation of the total GPT activity. A 10% homogenate in 0.25 M sucrose was used for enrichment of the particulate fractions by centrifugation in the usual way or by gradient centrifugation. Techniques such as freeze-thawing, sonication, and incubation with detergents are frequently used to increase the accessibility of mitochondrial enzymes to their substrates. Such treatments did not enhance GPT activity in mitochondria; hence, the mitochondria were suspended in buffer and assayed immediately.

GPT catalyzes the following reaction:

Activity of the enzyme was measured by incubating L-alanine and α -ketoglutarate with the sample at 30°. Aliquots were pipetted at intervals into 20% trichloroacetic acid and the protein was removed by centrifugation. An aliquot (now containing pyruvate) and sufficient phosphate buffer, pH 7.2, to bring the final volume to 3.0 ml were pipetted into a cuvette. α -Dihydrnicotinamide adenine dinucleotide (NADH) was added and the optical density was read in a Beckman DU spectrophotometer at 340 m μ and recorded on a Gilford Multiple Sample Absorbance Recorder. Lactic dehydrogenase was added and the change in optical density was recorded.

Under the conditions of the assay a decrease in optical density of 2.07 units is equivalent to the oxidation of 1 μ mole of NADH, and therefore, to the reduction of 1 μ mole of pyruvate. It was necessary to use an indirect assay method because the activity was low in the particulate fractions, and the presence of other enzymes and endogenous substrates gave rise to large blank values.

The reverse reaction was also measured indirectly using pyruvate and glutamate as substrates and measuring the α -ketoglutarate formed

with glutamic dehydrogenase and NADH. Concentrations of the various substrates which would give a zero-order reaction rate were determined by varying the amount of each substrate independently until optimum conditions obtained.

Protein concentration was measured by the biuret reaction, and specific activity was expressed as micromoles of product formed per minute per mg of protein.

Results and Discussion

Cytological Distribution

Enzyme assays carried out on the five fractions obtained by differential centrifugation showed a small amount of GPT activity in the nuclear (2.2% of the total), microsomal (1.2%), and "poorly sedimentable" (0.3%) fractions. Activity in the mitochondria was higher (4.7%) than in the other particulates, but most of the GPT activity was in the supernatant fraction (89%). Similar distributions have been reported by others,⁽¹⁻³⁾ although Segal et al.⁽⁴⁾ found no activity in the particulate fractions.

Because gradient centrifugation affords a cleaner fractionation of cellular components, this technique was used to show more clearly the presence of GPT in the mitochondria.* Succinic dehydrogenase, a mitochondrial enzyme, was used as a reference to show the precise location of peak mitochondrial activity. Figure 22 shows the distribution of GPT and succinic dehydrogenase activity in fractions of a whole homogenate; Figure 23 shows their distribution in a mitochondrial fraction isolated by differential centrifugation. In the latter the peak of GPT activity coincides with that of succinic dehydrogenase, indicating that GPT activity is definitely present in the mitochondria.

Figure 22

Distribution of GPT ($\Delta-\Delta$) and succinic dehydrogenase ($O-O$) after gradient centrifugation of liver homogenate

*We are grateful to Dr. John F. Thomson for performing the gradient centrifugations and the analysis of succinic dehydrogenase.

Figure 23

Distribution of GPT (Δ - Δ) and succinic dehydrogenase (O-O) after gradient centrifugation of the mitochondrial fraction of a liver homogenate

Metabolic Studies

A study of the effect of various dietary and hormonal treatments on GPT activity in the supernatant and mitochondrial fractions of rat liver was made to compare the responses of the transaminases in the two fractions. The isozymes reacted similarly to treatment with gluconeogenic hormones, but failed to do so in animals either kept on diets containing different amounts of protein or made diabetic (Table 19 and Figure 24). In all conditions that caused the breakdown of protein, whether of endogenous or exogenous origin, the activity of the soluble enzyme was consistently elevated. Conversely, in conditions that caused a reduced catabolism of protein, GPT activity in the supernatant fraction was lowered. These results are consistent with those reported by others.⁽⁵⁻⁹⁾ On the other hand, the mitochondrial enzyme did not respond in this manner in all cases, suggesting that the isozymes are different proteins and only partly under the same control.

TABLE 19
Metabolic studies of the isozymes of GPT

Treatment	No. of animals used	Change in body weight, g	Mitochondria			Supernatant		Homogenate		M + S**
			Protein, mg/g	Specific activity, μ moles/min/mg	Total units, μ moles/min	Specific activity, μ moles/min/mg	Total units, μ moles/min	Specific activity, μ moles/min/mg	Total units, μ moles/min	
Control: 12% Casein	8	-6	50.9	0.014 $\pm$ 0.002†	0.64 $\pm$ 0.07	0.248 $\pm$ 0.017	20 $\pm$ 1.0	0.081 $\pm$ 0.006	20 $\pm$ 1	20.6
2% Casein	6	-14	36.9	0.013 $\pm$ 0.001	0.48 $\pm$ 0.037	0.153 $\pm$ 0.021	11 $\pm$ 1.7	0.056 $\pm$ 0.008	12.2 $\pm$ 1.4	11.5
40% Casein	4	+8	63.9	0.027 $\pm$ 0.002	1.7 $\pm$ 0.137	0.366 $\pm$ 0.063	34.2 $\pm$ 5.83	0.115 $\pm$ 0.018	33.6 $\pm$ 3.4	35.9
Fasted	6	-40	50.1	0.015 $\pm$ 0.002	0.731 $\pm$ 0.123	0.369 $\pm$ 0.039	32.3 $\pm$ 3.07	0.147 $\pm$ 0.013	34.9 $\pm$ 2.89	33.0
Essential amino acids	4	-9	33.0	0.0053 $\pm$ 0.002	0.19 $\pm$ 0.09	0.068 $\pm$ 0.021	5.95 $\pm$ 0.81	0.032 $\pm$ 0.007	6.1 $\pm$ 1.55	6.1
Essential amino acids plus alanine	4	-3	34.0	0.0059 $\pm$ 0.003	0.21 $\pm$ 0.11	0.077 $\pm$ 0.022	6.68 $\pm$ 1.8	0.035 $\pm$ 0.007	7.24 $\pm$ 1.56	6.9
Prednisolone, 2 mg/rat/day	4	-58	46.5	0.057 $\pm$ 0.008	2.6 $\pm$ 0.360	0.955 $\pm$ 0.041	71.3 $\pm$ 6.96	0.353 $\pm$ 0.021	71.5 $\pm$ 3.9	73.9
Cortisone, 13 mg/rat/day	4	-26	49.9	0.033 $\pm$ 0.003	1.7 $\pm$ 0.16	0.619 $\pm$ 0.125	50.6 $\pm$ 6.43	0.229 $\pm$ 0.015	50.1 $\pm$ 5.1	52.3
DCA*, 10 mg/rat/day	4	-12	45.9	0.011 $\pm$ 0.003	0.52 $\pm$ 0.238	0.172 $\pm$ 0.019	14.6 $\pm$ 1.79	0.066 $\pm$ 0.011	13.7 $\pm$ 1.23	15.1
Alloxan, 175 mg/kg	4	-44	43.9	0.019 $\pm$ 0.001	0.818 $\pm$ 0.069	0.402 $\pm$ 0.052	32.4 $\pm$ 2.13	0.159 $\pm$ 0.020	33.4 $\pm$ 3.14	33.2

*Deoxycorticosterone.

**Mitochondria plus supernatant.

†Standard error of the mean.

Figure 24

Change in activity of mitochondrial and soluble isozymes in response to diets containing different amounts of protein, to treatment with hormones, to diabetes, and to fasting

In animals fed the different levels of protein, the changes in the mitochondrial protein (expressed as total protein per gram of fresh liver) roughly paralleled the change in activity of the mitochondrial isozyme (Table 19 and Figure 24). On the other hand, mitochondrial protein levels in animals treated with hormones were unaltered although the GPT activity changed markedly. It would appear that the mitochondrial population may be modified by protein level of the diet but not by changes in protein metabolism associated with gluconeogenesis.

Chemical Characteristics

Further evidence for the existence of two GPT proteins was obtained from the examination of the chemical characteristics of the isozymes prepared from rats injected with prednisolone. The supernatant or soluble enzyme was enriched according to purification procedure II of Segal et al.⁽⁴⁾ through the DEAE step. The first problem encountered in the isolation of the mitochondrial isozyme was that of solubilization of the enzyme. Freeze-thawing at either -195° or -10° , sonication, and treatment with detergents all failed to yield an appreciable amount of solubilized enzyme. However, when the mitochondria were first suspended in cold, 50% glycerol-0.001 M alanine, sedimented, and resuspended in 0.05 M phosphate buffer-0.025 M alanine, an active, soluble preparation of the enzyme could be obtained.

Further enrichment has so far been frustrated because of the marked instability of the enzyme. Whereas the soluble isozyme at all stages of purification was stable for weeks when stored at 0° , the mitochondrial form lost most of its activity in a few hours. Heat, pH changes away from neutrality, fractionation with ammonium sulfate, or dialysis led to rapid loss of activity. This loss was not the consequence of dissociation of the co-enzyme from the protein, since addition of, or incubation with, pyridoxal phosphate did not restore activity. The inactivation could be slowed somewhat by storage of the enzyme in a 50% glycerol solution or in 0.05 to 0.1 M alanine. It was necessary, therefore, to make preliminary estimates of the pH optima and apparent Michaelis constants (K_m) on the crude solubilized enzyme.

Assays for the reverse reaction were carried out on both enzymes and the ratios of the specific activities were compared (Table 20). Examination of the pH curves of optimum activity for both isozymes indicated that the two may have different pH optima (Figure 25). The soluble enzyme shows a broad peak of activity centering about pH 7.2; the mitochondrial enzyme shows a peak at about pH 8.0. These results are in contrast to those of Katunuma et al.(3) who reported optimum activity at pH 9.7 for the

TABLE 20

Comparison of forward and reverse reactions of the two isozymes

Mitochondria*	Forward reaction, μ moles pyruvate/ min/mg	Reverse reaction, μ moles α -ketoglutarate/ min/mg	Ratio: forward/reverse
From fractional centrifugation	0.11	0.04	2.75
Solubilized enzyme	0.013	0.016	0.81
Soluble			
From fractional centrifugation	0.32	0.24	1.33
Purified enzyme	12.4	9.49	1.30
Literature(10)			
Soluble (homogenate)			0.74

*These samples were not from the same animal and were assayed at different times.

Figure 25

Change in GPT activity of soluble and mitochondrial isozymes with varying pH

soluble enzyme and pH 8.5 for the mitochondrial isozyme. Segal et al.⁽⁴⁾ obtained a broad peak for the soluble (homogenate) enzyme ranging from pH 7.2-8.5. Absolute activity of the soluble enzyme in the different buffers varied widely, while that of the mitochondrial enzyme in Tris and phosphate buffers was nearly the same.

Kinetic constants obtained for the two enzymes are shown in Table 21. The apparent Michaelis constants for α -ketoglutarate are similar for the isozymes, but those for alanine are quite different. The absolute K_m for α -ketoglutarate for the soluble enzyme has been calculated to be 1.1 and that for alanine to be 34.⁽¹⁰⁾ The Michaelis constants for aspartic acid for the isozymes of glutamic-oxalacetate transaminase^(11,12) also differ greatly.

TABLE 21

Michaelis constants

Enzyme	α -ketoglutarate, mmoles	L-alanine, mmoles
Soluble	0.81	34.0
Mitochondria	0.68	5.0

When conditions for stabilization of the mitochondrial GPT are found, it may be possible to enrich the enzyme so that the kinetic and chemical properties of this isozyme may be studied more definitively.

Summary

Measurement of enzyme activity under various conditions of protein intake and corticosteroid administration showed that the enzymes do not always react in an identical manner to treatment of the host. Differences in the stability, methods of enrichment, pH optima, and kinetic constants strongly suggest that these are, indeed, isozymes of glutamic-pyruvic transaminase.

A new technique for solubilizing mitochondrial enzymes was developed; the technique involves osmotic shock after partial replacement of water in the mitochondria with glycerol.

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EFFECT OF ISOTOPE EXCHANGE ON THE PROTONATION OF DNA

Lyle G. Bunville

In an investigation of the intramolecular interaction of the bases in DNA, hydrogen-ion binding curves have been determined in the solvents H_2O and D_2O , each at an ionic strength of 0.10 in NaCl , 25°C . In the latter system the titration curve at low degrees of binding is shifted towards higher pH values by an amount ($\Delta\text{pH} = 0.42$) consistent with that expected from the equilibrium isotope effect on the dissociation constants of low molecular weight acids. As the degree of binding increases, ΔpH increases to a constant value of 0.52 throughout the region of the helix-coil transition. Upon completion of the transition, ΔpH again increases with the degree of binding. In each system the end point of the transition occurs at the same degree of binding, indicating that no significant change of stability results from the exchange of hydrogen for deuterium in the double helix, or that the stability of both helix and coil are altered to the same extent. The increasing displacement of the two binding curves with increasing degree of protonation is indicative of enhanced binding by DNA in D_2O , either by perturbation of the intrinsic dissociation constants of the bases, or by alteration of molecular dimensions.

NUCLEAR CHANGES IN RAT LIVER CELLS DURING THIOACETAMIDE ADMINISTRATION

A. D. Barton and Elizabeth A. Cerny*

Rather⁽¹⁾ has shown that the administration of thioacetamide to rats produces a remarkable increase in the volume of parenchymal cell nuclei in the liver and a striking increase in the size of the liver cell nucleoli. Laird⁽²⁾ has shown that in short-term administration of the drug, the increase in nuclear volume is not due to an increase in ploidy, since the average deoxyribonucleic acid (DNA) content per cell does not increase. Laird⁽²⁾ and others^(3,4) have demonstrated an increase in the ribonucleic acid (RNA) and protein of the enlarged nuclei. The present study was undertaken to learn more about these changes in RNA and protein, using a considerably improved method of isolating nuclei that was not available when the early studies were made.

Methods

The animals used were adult male Sprague-Dawley albino rats, body weight 300-425 g; Wayne "Lab-blox" (Allied Mills) and water were available ad lib. The animals in the treated groups received thioacetamide daily, (10 mg as a 1% solution in isotonic saline-0.18 mM CaCl₂) injected subcutaneously in the scapular region. The treatment periods were 4, 7, 11, and 14 days. At each time point, a total of three treated groups and one control group that had received corresponding injections of the salt solution with the thioacetamide omitted, were sacrificed for study. A zero-time control group received no injections. For each group, four rats were anesthetized with ether, and the livers were removed and transferred to a cold room maintained at 2-4°C; perfusion of the livers with isotonic saline-0.18 mM CaCl₂ and all subsequent operations were carried out at cold room temperature.

From the pooled livers, one representative sample was taken to prepare a 10% homogenate in 30% sucrose, and from this homogenate, aliquots were taken for nuclear counts and for analysis to determine RNA and DNA by Schneider's method.⁽⁵⁾ A Cooke image-splitting eyepiece was used to measure nuclear diameters; a total of 36-60 nuclei were measured at each time point.

A second representative sample was taken from the pooled livers to prepare a 10% homogenate in 75% sucrose-0.18 mM CaCl₂, from which the liver cell nuclei were isolated by a modification of the method of Chaveau et al.⁽⁶⁾ as described previously.⁽⁷⁾

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The "soluble" nuclear proteins were obtained by extracting the isolated nuclei with 0.14 M NaCl-0.18 mM CaCl₂ and centrifuging the extracted nuclei.⁽⁷⁾ The RNA and DNA contents of the nuclear sediment were determined after extraction with hot trichloroacetic acid.⁽⁵⁾ The residue was washed twice with 95% ethanol and twice with ether; it was then dried to constant weight and the "residue" protein was determined gravimetrically.

Results

The results of the determinations made on the various preparations are given in Table 22. There was little change in cell size (as indicated by the number of nuclei per gram of liver) or in the amount of DNA per nucleus or in the amount of RNA per cell or per gram of tissue. The increase in nuclear volume was statistically significant ($P = 0.01$) by the eleventh day of thioacetamide administration.

TABLE 22
Changes in rat liver cells with daily administration of thioacetamide

Preparation	Control n = 5	4 days n = 3	7 days n = 3	11 days n = 3	14 days n = 3
Whole Tissue					
Nuclei/g liver ($\times 10^{-6}$)	131 $\pm$ 4**	138 $\pm$ 5	140 $\pm$ 7	120 $\pm$ 3	122 $\pm$ 3
DNA (mg/g liver)	1.97 $\pm$ 0.06	1.99 $\pm$ 0.03	1.82 $\pm$ 0.04	1.69 $\pm$ 0.01	1.57 $\pm$ 0.08
DNA/cell ($\text{g} \times 10^{-11}$)	1.51 $\pm$ 0.06	1.45 $\pm$ 0.07	1.30 $\pm$ 0.05	1.41 $\pm$ 0.10	5.30 $\pm$ 0.08
RNA (mg/g liver)	5.14 $\pm$ 0.15	5.84 $\pm$ 0.24	5.10 $\pm$ 0.15	4.71 $\pm$ 0.10	5.30 $\pm$ 0.08
RNA/cell ($\text{g} \times 10^{-11}$)	3.94 $\pm$ 0.09	4.24 $\pm$ 0.05	3.65 $\pm$ 0.12	3.92 $\pm$ 0.02	4.34 $\pm$ 0.04
Nuclei					
RNA/DNA	0.194 $\pm$ 0.016	0.278 $\pm$ 0.021	0.395 $\pm$ 0.005	0.431 $\pm$ 0.020	0.407 $\pm$ 0.026
"Residue" protein/DNA	3.30 $\pm$ 0.12	3.52 $\pm$ 0.29	4.78 $\pm$ 0.02	4.66 $\pm$ 0.15	4.38 $\pm$ 0.22
"Soluble" protein/DNA	1.19 $\pm$ 0.08	1.07 $\pm$ 0.15	1.15 $\pm$ 0.07	1.07 $\pm$ 0.08†	1.21 $\pm$ 0.10
Nuclear volume (μ^3)	1043 $\pm$ 70	1625 $\pm$ 158	1359 $\pm$ 162	1945 $\pm$ 212	1933 $\pm$ 200

* Number of preparations on which determinations were made.

** Mean $\pm$ standard error.

† $n = 2$.

In the isolated nuclei, the ratio of RNA to DNA increased by more than 100%, the increase being significant at the 0.01 level by the seventh day of treatment. The ratio of "residue" protein to DNA increased by nearly 50%, and again the increase was statistically significant by the seventh day of treatment. There was no significant change in the ratio of "soluble" protein to DNA throughout the course of thioacetamide treatment.

Discussion

The increases in nuclear volumes, and the lack of change in cell size, or in the amount of DNA per nucleus, or in the amount of RNA per cell or per gram of liver, are all in agreement with the results of earlier studies.^(2,4) The increases in nuclear RNA and protein reported previously^(2,4) are confirmed by the present results with nuclear preparations containing considerably less contaminating (non-nuclear) material.⁽⁶⁻⁸⁾

By studying the "soluble" (saline-extractable) and "residue" protein separately, it has been possible to show that the increase in protein is entirely in the "residue" protein fraction. Our previous studies showed that the saline-extractable proteins are retained in the nucleus by ion binding, presumably to nucleic acid, since they are basic proteins. (9,10) In the present experiments, there was no increase in saline-extractable protein despite the large increase in nuclear RNA; this result, together with the low ratio of RNA to DNA in the isolated nuclei indicates that these saline-extractable proteins are probably associated with the DNA, rather than RNA in the nucleus, i.e., with the chromosomes. Although the thioacetamide treatment led to considerable increases in both protein and RNA in the liver nuclei, it did not produce any quantitative change in the capacity of the chromosomes to bind these soluble proteins.

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NUCLEAR CHANGES IN RAT KIDNEY CELLS AFTER CASTRATION AND ADMINISTRATION OF TESTOSTERONE

A. D. Barton and Kathleen M. Tracy*

Rabinovitch and Valeri,⁽¹⁾ studying histological sections, have reported that castration of mice leads to a decrease in the volume of the nuclei in the cells of the kidney, and that the nuclear volume increases when testosterone is administered. Bern and Alfert,⁽²⁾ using quantitative microspectrophotometry on stained histological sections, showed that the increase in nuclear volume was not due to an increase in ploidy. Their results indicated an increase in the nonhistone protein content of the enlarged nuclei. In the present study, kidney cell nuclei have been isolated from animals in which the testosterone balance was altered in various ways. The changes in nuclear constituents that accompany changes in the level of testosterone activity have been followed by direct measurements on the isolated nuclei.

The animals used were adult, male, Sprague-Dawley albino rats; age 3-4 months; weight 250-300 g. Wayne "Lab-blox" (Allied Mills) and water were available ad lib. In addition to controls, animals were examined in four experimental conditions: castrate; castrate treated with testosterone for 4 days; same treated for 8 days; and normal treated with testosterone for 6 days. Castration was performed under ether anesthesia.⁽³⁾ The castrates were killed for study 17-30 days after the operation, and the corresponding controls were sacrificed as close to the same age as the schedule of experiments would permit. The hormone treatment consisted of a daily subcutaneous injection of 1 mg testosterone (Lilly).

The animals were sacrificed by cervical dislocation under ether anesthesia. The kidneys were removed and perfused with cold isotonic saline in a cold room at 2-4°C, where all subsequent operations were carried out; the kidneys from 8 animals were pooled for each preparation.

From the pooled kidneys, a representative sample was taken to prepare a 10% homogenate in 30% sucrose, and from this homogenate aliquots were taken for the determination of RNA and DNA by Schneider's method.⁽⁴⁾ For nuclear counts and measurement of nuclear diameters in the Cooke image-splitting eyepiece, aliquots of the homogenate were diluted 1:60 with 2% acetic acid containing gentian violet; diameters of 30-40 nuclei were measured for each experimental condition.

A second representative sample was taken from the pooled kidneys to prepare a 10% homogenate in 75% sucrose-0.18 mM CaCl₂, from which the kidney cell nuclei were isolated by the modification of the method of Chaveau et al.⁽⁵⁾ used previously for the isolation of liver cell nuclei.⁽⁶⁾

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The "soluble" (saline-extractable) nuclear proteins were obtained by extracting the isolated nuclei with 0.14 M NaCl-0.18 mM CaCl₂ and centrifuging the extracted nuclei.(6) The RNA and DNA contents of the nuclear sediment were determined after extraction with hot trichloroacetic acid. The residue was washed with 95% ethanol and twice with ether, and the "residue" protein was determined gravimetrically.

The weight of the seminal vesicles dropped sharply after castration and increased markedly with administration of testosterone (Table 23); most of the changes are significant at the 0.001 level. Throughout the various groups, increased levels of testosterone produced a consistent trend towards increased kidney weight and a lower concentration of DNA per gram of kidney, but even between the extreme conditions the changes were not large and were barely significant at the 0.05 level. The product of these two measures, which gives the total DNA per kidney, showed no consistent changes. In agreement with a previous report,(7) there was no significant change in the amount of RNA per gram of kidney, which implies that the total RNA per kidney increases with testosterone administration.

TABLE 23

Changes in rat seminal vesicles and kidney cells in response to castration and administration of testosterone

	Normal n* = 3	Castrate n = 5	Castrate + Testosterone		Normal + Testosterone 6 days n = 2
			4 days n = 3	8 days n = 3	
<u>Seminal Vesicles</u> weight, g	1.40 ± 0.12**	0.13 ± 0.02	0.58 ± 0.08	1.72 ± 0.22	2.20 ± 0.06
<u>Kidneys</u>					
<u>Whole Tissue</u> weight, g	2.95 ± 0.10	2.72 ± 0.07	2.92 ± 0.07	3.07 ± 0.04	3.21 ± 0.18
DNA, mg/g kidney	3.94 ± 0.12	4.46 ± 0.20	3.58 ± 0.30	3.77 ± 0.23	3.64 ± 0.14
RNA, mg/g kidney	3.70 ± 0.13	3.77 ± 0.21	3.27 ± 0.24	3.89 ± 0.31	3.72 ± 0.08
<u>Nuclei</u>					
Nuclear volume, μ ³	575 ± 30	472 ± 19	504 ± 29	462 ± 30	705 ± 51
RNA/DNA	0.092 ± 0.010	0.094 ± 0.006	0.084 ± 0.006	0.089 ± 0.005	0.094 ± 0.008
"Residue" protein/DNA	2.32 ± 0.08	2.72 ± 0.15	2.34 ± 0.08	2.17 ± 0.42	2.10 ± 0.16
"Soluble" protein/DNA	0.492 ± 0.004†	0.603 ± 0.058	0.570 ± 0.016	0.472 ± 0.011	0.414 ± 0.023

*Number of preparations on which determinations were made; kidneys from 8 animals pooled for each preparation.

**Mean ± standard error.

†n = 2.

Castration reduced the nuclear volume in the kidney cells and testosterone treatment increased it. The nuclear volume in the castrates was significantly different at the 0.01 level from that in the normals or in the testosterone-treated normals. There was no significant change in the ratio of RNA to DNA in the kidney cell nuclei. Increasing levels of testosterone were accompanied by a consistent trend towards a decrease in the ratio of both "residue" protein to DNA and "soluble" protein to DNA in the isolated nuclei. Regression analysis indicated that this trend is significant at the 0.05 level in the case of the "residue" protein and is not significant in the case of the "soluble" protein.

The drastic changes in the weight of the seminal vesicles indicate that the various treatments were altering the testosterone balance effectively. The trend toward increased kidney weight and nuclear volume with increasing levels of testosterone confirmed previous reports.^(1,2,7) These results, together with the decrease in DNA concentration without change in the total amount of DNA, suggest that the effect of testosterone is to increase the size of the kidney cells and their nuclei, without changing the total number of cells present.

The work of Bern and Alfert⁽²⁾ indicated a decrease in the concentration of histone upon administration of testosterone, due to dilution from enlargement of the nuclei; the concentration of nonhistone protein in the nucleus remained the same and, therefore, the total amount was assumed to have increased, since the nuclear volume increased. In the present experiments, the total amount of protein in the isolated nuclei was higher in the absence of the hormone, and decreased with testosterone, despite the fact that nuclear volume increased. The difference in results is probably related to the different methods used. Bern and Alfert measured protein in fixed tissue sections, which presumably included all of the protein present in the nuclei.⁽⁸⁾ Our isolated nuclei contained only those proteins that were bound firmly enough to be retained during the isolation procedure.⁽⁹⁾ Thus, while the increase in nuclear volume produced by testosterone administration was accompanied by an increase in the proteins that were bound very loosely, if at all, in the nucleus, our results indicated that there was a slight decrease in the proteins that are more firmly bound, and little, if any, change in the saline-extractable protein of the nuclei.

Comparing the nuclear enlargement produced by thioacetamide administration⁽¹⁰⁾ with that produced by increased levels of testosterone activity, we find some similarity and some distinct differences. With thioacetamide administration, there is a prominent increase in the size of the nucleoli, and there is a large increase in the amount of nuclear RNA and "residue" protein. In the nuclear enlargement produced by testosterone administration, there is no obvious change in nucleolar size, no increase in nuclear RNA, and, if anything, a slight decrease in "residue" protein. Neither treatment produced any quantitative change in the capacity of the chromosomes to bind the saline-extractable proteins.

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

John F. Thomson and Sharron L. Nance

The previous report in this series was concerned with studies on the capacity of mitochondrial fractions isolated from livers of irradiated rats to esterify inorganic phosphate, using the energy derived from the oxidation of fumarate or α -ketoglutarate.⁽¹⁾ The data indicated no consistent effect of X-irradiation on either phosphorylative capacity or phosphorylative efficiency, in contrast to the earlier observations of Hall et al.⁽²⁾

This report is concerned with additional studies carried out with male rats (instead of the females used in most of this work), with the effect of fluoride on α -ketoglutarate oxidation, and with β -hydroxybutyrate as the substrate.

Methods

In most experiments, Badger Research Sprague-Dawley female rats, 2 to 4 months old, were used; one series of experiments was carried out with male rats of the same strain, 11 months old. Irradiated rats received 1000 r total-body X-radiation at a dose rate of 200 r/min for females and 100 r/min for males; they were killed $3\frac{1}{2}$ to 4 hr after exposure.

The procedure for isolation of mitochondrial fractions was the same as that described previously.⁽¹⁾ Four mitochondrial fractions were obtained from each liver.

Results and Discussion

Table 24 is a summary of data, obtained under a variety of experimental conditions with female rats, on oxygen consumption, phosphate esterification, P/O ratios, and total nitrogen in different mitochondrial fractions. The data from the previous report are included in this table. The same measurements, carried out on preparations from male rats with α -ketoglutarate as substrate, are shown in Table 25.

Among the 72 comparisons made in these two tables, only two significant differences were observed, both with respect to oxygen consumption with α -ketoglutarate as the substrate: Zone B, female rats, fluoride present; and Zone A, male rats. As far as phosphorylative efficiency (P/O) and capacity (P/N) were concerned, there was not even a trend to show an effect of irradiation; in fact, in eleven of the 21 comparisons, both the P/O and P/N ratios were slightly higher after irradiation.

TABLE 24

Oxidative phosphorylation in mitochondria prepared from livers of control and irradiated female rats

Zone*	A			B			C			D ₁			D ₂		
Size range (μ)	0.37-0.55			0.55-0.69			0.69-0.82			0.82-0.94			>0.82		
	N	Av.	σ	N	Av.	σ	N	Av.	σ	N	Av.	σ	N	Av.	σ
α -ketoglutarate + malonate, F ⁺ present															
P/N** Control Irradiated	11 11	18.2 17.6	0.86 1.46	11 11	27.9 26.2	0.68 0.61	11 11	29.2 28.3	0.76 0.85	7 6	30.2 28.7	1.14 1.99	4 4	33.1 28.5	2.03 3.23
Q/N† Control Irradiated	11 10	5.60 5.93	0.256 0.521	11 11	7.46 6.69	0.200 0.234	11 11	8.26 7.58	0.374 0.310	6 6	7.48 7.67	0.278 0.500	4 4	8.52 7.58	0.703 0.578
P/O†† Control Irradiated	12 10	3.26 3.01	0.133 0.245	12 11	3.67 3.86	0.089 0.098	12 11	3.65 3.82	0.077 0.090	7 5	3.94 3.85	0.139 0.076	4 4	3.91 3.65	0.167 0.093
α -ketoglutarate + malonate, F ⁻ omitted															
P/N Control Irradiated	3 5	10.8 11.4	1.33 1.39	4 5	26.8 25.2	1.45 1.40	4 5	28.2 29.7	0.97 1.12	4 5	28.2 29.7	0.97 1.12	4 5	21.9 24.1	0.35 2.89
Q/N Control Irradiated	4 5	6.28 5.71	0.540 0.508	4 5	8.24 8.41	0.247 0.364	4 5	8.95 8.91	0.414 0.467	4 5	8.95 8.91	0.414 0.467	4 5	7.57 8.13	0.383 0.680
P/O Control Irradiated	3 5	1.81 1.96	0.242 0.071	4 5	3.13 3.02	0.191 0.159	4 5	3.16 3.35	0.170 0.106	4 5	3.16 3.35	0.170 0.106	4 5	2.93 2.97	0.179 0.153
Fumarate, F ⁺ present															
P/N Control Irradiated	7 7	12.2 13.1	0.80 0.50	7 7	15.8 16.8	0.86 1.41	7 7	16.6 17.9	1.71 0.81	7 7	13.5 15.9	1.11 1.05	7 7	10.1 11.1	0.84 0.50
Q/N Control Irradiated	8 5	4.97 5.51	0.488 0.567	8 5	5.59 6.12	0.357 0.531	8 5	6.12 6.12	0.375 0.342	7 5	5.33 6.09	0.345 0.576	7 5	4.35 4.92	0.098 0.614
P/O Control Irradiated	6 5	2.44 2.50	0.128 0.179	7 5	2.78 2.87	0.127 0.149	7 5	2.78 2.97	0.060 0.101	7 5	2.57 1.18	0.118 0.194	7 5	2.32 2.31	0.182 0.157
β -hydroxybutyrate, F ⁺ present															
P/N Control Irradiated	5 4	14.1 13.3	0.54 0.81	5 4	12.6 13.3	0.48 0.94	5 4	12.2 13.2	0.84 1.01	5 4	12.2 13.2	0.84 1.01	5 4	10.1 11.1	0.84 0.50
Q/N Control Irradiated	5 4	6.23 5.50	0.260 0.344	5 4	5.48 5.72	0.239 0.663	5 4	5.35 5.88	0.308 0.481	5 4	5.35 5.88	0.308 0.481	5 4	4.35 4.92	0.098 0.614
P/O Control Irradiated	5 4	2.26 2.54	0.026 0.202	5 4	2.31 2.34	0.080 0.099	5 4	2.29 2.32	0.210 0.066	5 4	2.29 2.32	0.210 0.066	5 4	2.32 2.31	0.182 0.157
Nitrogen content (composite of above experiments)															
mg N/g liver Control Irradiated	29 27	0.89 0.90	0.032 0.031	29 27	1.38 1.34	0.039 0.053	29 27	1.47 1.38	0.046 0.033	15 14	0.82 0.74	0.043 0.152	14 13	2.98 2.78	0.218 0.187

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

**Micromoles P esterified/mg N/20 min.

†Micromoles O consumed/mg N/20 min.

††Micromoles P esterified/micromoles O consumed.

Our work on the effects of radiation on the phosphorylative efficiency of rat spleen mitochondria⁽³⁾ indicated that a radiation-induced depression of the P/O ratio could be observed only when experimental conditions were such that control preparations were unable to esterify phosphate efficiently.

However, in these experiments with liver, low P/O ratios were observed in control rats under a variety of conditions - throughout the series

with β -hydroxybutyrate, in Zone A with all substrates, and in all zones with α -ketoglutarate when fluoride was omitted - but no further depression of the P/O ratio was noted after total-body irradiation. Hence the explanation offered to account for the occasional decrease in P/O ratios of irradiated spleen mitochondria, i.e., a relative increase in mitochondrial contaminants with high phosphatase activity, (3) would seem not to apply in these studies.

TABLE 25

Oxidative phosphorylation in mitochondria prepared from livers of control and irradiated male rats*

Zone	A			B			C			D ₂		
Size range (μ)	0.37-0.55			0.55-0.69			0.69-0.82			>0.82		
	Av.	σ	p	Av.	σ	p	Av.	σ	p	Av.	σ	p
P/N												
Control	10.0	1.33	} 0.6	19.9	0.79	} 0.8	21.2	0.78	} 0.9	14.3	2.27	} 0.9
Irradiated	8.9	1.75		20.1	1.01		21.0	1.28		14.4	1.34	
O/N												
Control	4.41	0.301	} 0.02	6.09	0.252	} 0.4	5.71	0.180	} 0.7	4.84	0.321	} 0.3
Irradiated	2.99	0.343		5.74	0.345		5.89	0.391		4.36	0.329	
P/O												
Control	2.28	0.271	} 0.2	3.27	0.018	} 0.1	3.70	0.062	} 0.5	2.94	0.365	} 0.5
Irradiated	2.92	0.395		3.56	0.124		3.59	0.141		3.26	0.237	
mg N/g liver												
Control	0.91	0.060	} 0.2	1.63	0.169	} 0.4	1.60	0.123	} 0.7	3.41	0.311	} 0.7
Irradiated	0.83	0.032		1.47	0.091		1.66	0.102		3.28	0.157	

* α -ketoglutarate as substrate. Each value is the average of 5 rats; see Table 24 for units.

The discrepancy between our results and those of Hall et al. (2) is still unresolved. Since we have observed no radiation effects with mitochondria isolated from Sprague-Dawley or Holtzman rats, males or females, assayed with different substrates with or without fluoride, the only plausible explanation remaining is an unrecognized variation in experimental technique.

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PHOSPHOLIPASE C, LOW pH, AND TEMPERATURE EFFECTS ON ACID PHOSPHATASE RELEASE FROM RAT LIVER, SPLEEN AND THYMUS

Y. E. Rahman

Introduction

The fact that "lysosomes" are a special group of particles, morphologically and functionally distinct from other subcellular particles, has been indisputably established during the course of the past few years. However, studies on lysosomes have been limited mainly to rat liver tissue, and very little is known about the functional and physiological characteristics of lysosomes in other tissues, although their existence in a number of other tissues has been demonstrated.

A study on the effect of X-irradiation on the fragility of rat spleen lysosomes was reported earlier.^(1,2) The present report is concerned with acid phosphatase release from rat liver, spleen, and thymus lysosomes under the effects of phospholipase C, low pH, and different temperatures.

Methods

Tissue preparation and acid phosphatase assay are essentially the same as described previously,^(2,3) except that the 0.25 M sucrose used for homogenization contained 0.05 M tris-maleate buffer, at pH 7.0 or pH 5.2 as indicated for each experiment. The filtered tissue homogenate was then incubated at 0°, 25°, or 37°C, and aliquots of the homogenate were taken for assay of free acid phosphatase activity at 10, 30, 60, 120, and 180 minutes. At the end of each experiment, another aliquot of the tissue homogenate was taken for assay of total activity, using 0.1% Igepal-630, a non-ionic detergent, which, at this concentration, causes release of all the acid phosphatase bound in the lysosomes. All free activities are calculated as per cent of the total enzyme activity.

Phospholipase C was purchased from Sigma Chemical Co.; the amount used for each experiment was dissolved in 0.25 M sucrose before addition to the tissue homogenate.

Results

Effect of Low pH and Temperature on Acid Phosphatase Release

Figure 26 shows the release of acid phosphatase from liver lysosomes incubated at 0°C and 37°C for times up to 3 hr. At 0°C, acid

phosphatase release did not change up to 3 hr, but at 37°C, this release is shown to increase with time, and approached 100% after 3 hr.

Figure 26

Effect of temperature on time-course of acid phosphatase release from liver lysosomes

Figures 27 and 28 show the result of the same experiment on spleen and thymus; here, contrary to the release of acid phosphatase from liver, even at 37°C the amount of released enzyme is practically unchanged for more than 3 hr.

Figure 27

Effect of temperature on time-course of acid phosphatase release from spleen lysosomes

Figure 28

Effect of temperature on time-course of acid phosphatase release from thymus lysosomes

Effect of Low pH, Temperature and Phospholipase C on Acid Phosphatase Release

Figures 29-31 show that the addition of phospholipase C at 25°C and pH 7.0 did not increase the release of acid phosphatase from liver lysosomes for 2 hr or from spleen and thymus lysosomes for 3 hr. When the pH was lowered to 5.2, however, the release of acid phosphatase increased with time. This release reached 100% at about 2 hr for the liver and the thymus but not more than 80% for the spleen, under the same conditions.

Figure 29

Effect of pH on time-course of acid phosphatase release from phospholipase C-treated liver lysosomes at 25°C

Figure 30

Effect of pH on time-course of acid phosphatase release from phospholipase C-treated spleen lysosomes at 25°C

Figure 31

Effect of pH on time-course of acid phosphatase release from phospholipase C-treated thymus lysosomes at 25°C

Figures 32 and 33 show that in both liver and spleen at pH 5.2 and treated with phospholipase C, but incubated at 37°C, acid phosphatase release is more rapid than at 25°C. Here again, the spleen failed to show complete release at this low pH, whereas at neutral pH, the acid phosphatase release is 100% at about 2 hr.

Figure 32

Effect of pH on time-course of acid phosphatase release from phospholipase C-treated liver lysosomes at 37°C

Figure 33

Effect of pH on time-course of acid phosphatase release from phospholipase C-treated spleen lysosomes at 37°C

Discussion

Previous studies on activation of lysosomal enzymes have been done mostly with rat liver, and from the results thus obtained, de Duve speculated that a possible mechanism of lysosome rupture in intact cells was the lowering of the intracellular pH.⁽⁴⁾ In view of the results reported here on spleen and thymus, we are not justified in extending this speculation to tissues other than liver.

The results obtained with phospholipase C seem rather too complicated to permit a conclusion at this moment; more experiments are needed before we will be able to understand the mechanisms of lysosome rupture in different mammalian tissues.

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FINE STRUCTURE OF THE LACUNAE AND CANALICULI IN BONE

F. Wassermann and James A. Yaeger

From light and electron microscopic observations and histochemical staining, it is known that osteocytes situated in their lacunae are surrounded by a layer of unmineralized material, the so-called capsule of the osteocytes.^(1,2,3) In a previous report⁽⁴⁾ the fine structure of this capsule was described as it appeared in the electron microscope after osmium fixation and demineralization. It consisted of a rather structureless, sometimes flocculent material, occupying a space of varying width between the surface of the osteocyte and the matrix of the bone. It was separated from the matrix by an osmiphilic layer about 30 m μ in diameter. This layer was not visible in sections of undemineralized bone, which indicated that it was occupied by bone crystals, called "coastal crystals."

There remained, however, some doubt as to whether the sometimes flocculent material of the capsule represented, as was suggested, connective tissue ground substance and, being continuous with the ground substance within the Haversian canals, could be considered the main route of transport between the blood and the bone matrix. Since osmium tetroxide fixation did not preserve sufficiently the fine structure of the osteocyte in demineralized bone, and in particular did not preserve its plasma membrane, one might suspect the flocculent material around the osteocyte of arising from disintegration of the periphery of the cell. Thus, we thought it desirable to apply to bone other fixing agents in addition to osmic acid in an attempt to confirm and improve the previous results. In the case of bone, it has turned out that the use of different fixatives is not only desirable but necessary, since no single fixative preserves all the structural details equally well.

Materials and Methods

All of the bone was chipped from the mid-shaft of the femur. Bone from mature dogs, guinea pigs and rats was fixed in buffered osmium tetroxide solution and decalcified in disodium ethylenediaminetetraacetate (EDTA), either with or without buffering to pH 7.3. Bone from mice about three weeks old was fixed in Luft's potassium permanganate solution,⁽⁵⁾ Tahmisian's permanganate-osmium fixative,⁽⁶⁾ or in glutaraldehyde⁽⁷⁾ followed by permanganate-osmium. This material was not demineralized. Finally, bone from young mice and mature rats was fixed in glutaraldehyde, demineralized in EDTA at pH 7.3, and postfixed in potassium permanganate-osmic acid. All of the material was embedded in Epon and sectioned on the Porter-Blum microtome with a glass knife.

Observations

Figure 34 represents a section of mature rat bone fixed in buffered osmium tetroxide and demineralized in EDTA. It shows an osteocyte in its lacuna surrounded by the pericellular space, and the osmiophilic layer adjacent to the bone matrix, which is characterized by the laminae of cross-banded collagen fibrils. Compared with the cell in Figure 37, this osteocyte is not well fixed. Since the membrane is not clearly defined, the few structures in the pericellular space could be particles of cellular origin.

Figure 34. Bone from femur of adult rat fixed in buffered osmium tetroxide, demineralized in EDTA. Section stained with uranyl acetate.

a. osteocyte cytoplasm; b. pericellular space; c. osmiophilic layer.

The material of Figure 35 was prepared also from the femur of mature rat and was fixed and stained in the same way as that of Figure 34; it presents again the conspicuous osmiophilic layer, which always appears in this way after osmium fixation. Figure 35 shows more clearly than Figure 34 a zone of very loose texture between the osmiophilic layer and the rest of the decalcified matrix. In the matrix the cross-banded fibrils are thicker and more densely packed than in the loose-textured zone. In the space between the osteocyte and the osmiophilic zone a few fibrils may be seen.

Figure 35. Bone from femur of adult rat, prepared as in Figure 34. Arrows indicate collagen fibrils in pericellular space.

The osteocyte in Figure 36 demonstrates the superiority of potassium permanganate fixation for revealing the membrane structures of the cell, including the plasma membrane, although the nucleic acids seem to be washed out (no ribosomes or other dense materials are seen in the nucleus). The osmiophilic layer and the loosely structured zone beyond it are not visible, since the bone was not demineralized. The edges of both cell and matrix are so well defined that the reality of the space around the cell (which is certainly not shrunken) cannot be doubted. The suggestion, made repeatedly in the past, that the osteocyte fills the lacuna completely in living bone is no longer tenable in view of these electron microscopic findings. Potassium permanganate fixation has also preserved numerous fibrils within the pericellular space; these are not visible in the light microscope. They are of varying diameters, the thicker ones measuring about 250 Å in width, and they are clearly cross-banded. The large body and well-developed cytoplasmic structures indicate that this osteocyte was recently encased and can be considered a "young" osteocyte. Cytoplasmic activity, suggested by the size and structure of the cell, seems to be indicated also by the small blebs which are seen in many places on the surface.

Figure 36. Bone from femur of two-week-old mouse fixed in permanganate. Section stained with lead citrate. Arrows indicate collagen fibrils in pericellular space.

Judged by the nuclear-cytoplasmic ratio, the osteocyte of Figure 37 is also "young." The section is from bone of a young mouse fixed in glutaraldehyde, demineralized, and postfixed in permanganate-osmium. This fixative preserves the nuclear and cytoplasmic structures and the cell membrane excellently, but the collagenous material in both the matrix and the capsule of the osteocyte is not as clearly defined as in the osmium-fixed specimens. It is of special interest that the dark line that stands out in the osmium-fixed material is represented in Figure 37 (and also in Figure 38) by a corresponding layer whose electron density is somewhat higher than that of the matrix and the capsule. The reality of the layer that contains the "coastal crystals" has thus been demonstrated by two different fixing methods.

The specimen in Figure 38 shows again the characteristics of glutaraldehyde and permanganate-osmium fixation. There are, however, significant differences between this case and that of Figure 37: the cytoplasmic body of the osteocyte is relatively small and the cell surface is smooth, which indicates that this cell is not as active as that in Figure 37;

Figure 37. Bone from femur of two-week-old mouse fixed in glutaraldehyde, demineralized in EDTA, postfixed in permanganate-osmium. Section stained with lead citrate. Arrows indicate osmiophilic layer. The pericellular space is inside this layer.

Figure 38. Bone from femur of adult rat prepared as in Figure 37. Arrows indicate pericellular space.

the pericellular space is very narrow and does not contain fibrils; and, finally, there is a broad zone between the outline of the pericellular space and the matrix proper. This zone corresponds to the loosely structured zone in the osmium-fixed specimens, where it is more conspicuous but probably not as well fixed as in the glutaraldehyde preparation. Since this layer has now been shown by two fixing methods, its reality seems certain. Since the osteocyte in Figure 35 is from the femur of an adult rat we feel justified in assuming that the differences between the osteocytes and their surrounding structures in Figures 37 and 38 are due to a difference in the ages of the cells.

In order to show the variability one encounters in sampling osteocytes from different materials, the osmic-acid fixed cell in Figure 39 is presented. In this case the space around the cell is filled with an unusually large number of relatively thick fibrils. The situation suggests that under certain, as yet unknown, conditions the osteocyte may function as a fibroblast. That the fibrils of the capsule are at some places continuous with those of the matrix and that the loosely structured zone adjacent to the osmiophilic layer is missing may be related to the abundant fiber formation.

Figure 39. Bone from femur of adult rat prepared as in Figure 34.

- a. large amount of collagen in pericellular space;
- b. canalliculi

In Figure 39, two processes of an osteocyte (indicated by arrows) are seen in their canaliculi. We want to emphasize that in no case have fibrils been observed in the canaliculi, although the other structures described in the lacunae were present. It is also important that in all canaliculi observed, including those less than $1\ \mu$ in diameter, cell processes could be identified.

Conclusions

1. It has been shown that more complete information about the fine structure of bone can be obtained by combining the results of several fixing agents. In this respect, the experience gained in the present study may be valuable for electron microscopic investigation of any biological structure.
2. The demonstration of a distinctive layer of greater electron density than either the bone matrix or the capsule of the osteocyte in glutaraldehyde-fixed bone shows that the osmiophilic layer previously described⁽²⁾ is not artificially produced by deposition of osmic acid at the edge of the matrix but represents a lamina existing in vivo that is a part of the transport system between the blood and the bone matrix.
3. It was also confirmed that the space surrounding the osteocyte corresponds to the intercellular material of connective tissue, and that it is not correct to characterize it as tissue fluid, as has been done in the past. This has been shown definitively by the demonstration of collagenous fibrils within the space. It is, therefore, certain that the interfibrillar material of the capsule of the osteocyte represents in bone, as in any other connective tissue, the medium of transport between the blood and both the cells and the matrix.
4. Since the smallest canaliculi were found to contain cytoplasmic processes of the osteocytes, it is not correct to assume that, as a rule, the osteocyte retracts its processes.
5. The formation of fibrils within the lacunae, which can be so abundant as to amount to a kind of fibrosis, shows that the osteocyte, after its enclosure in the lacuna, retains at least in part the activity of the osteoblast.
6. The zone outside the osmiophilic lamina, which differs in structure from the rest of the matrix, may well consist of bone that was laid down secondarily after the enclosure of the osteoblast. This point needs further investigation. If such secondary mineralization actually occurs, one must conclude that the distinction between osteoblast and osteocyte is, from the functional point of view, a rather arbitrary one; for the change in function seems to be gradual and possibly reversible.

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VARIOUS ASPECTS OF FIXATIVES FOR ULTRATHIN SECTIONS AND RESOLUTION IN ELECTRON MICROSCOPY

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There are two major requirements for a fixative used in electron microscopy: first, it must impregnate the tissue with a heavy element which will absorb electrons under the beam in proportion to its deposition; second, it must produce minimum distortion of the tissue. Osmium, permanganate, dichromate, and certain aldehydes, such as formalin and glutaraldehyde, are at present used for fine fixation. The first three are oxidizing agents that are reduced by the tissue. Their metallic elements form complexes with the unsaturated bonds of subcellular reductants. These heavy elements are able to absorb electrons more efficiently than the tissue itself and thus provide contrast for observing and photographing the specimen. Additional contrast may be gained by further staining. The aldehydes act as protein-denaturing compounds, but since organic compounds are not by themselves electron absorbers, the aldehyde-fixed tissue must later be stained with a heavy element-containing compound such as uranyl acetate, osmium tetroxide, lead citrate, potassium permanganate, gold chloride or phosphotungstic acid.

In order to obtain fine fixation, we have found that the tonicity of the fixing agent should be equivalent to isotonic saline.^(1,2) Some of the metallic compounds are relatively insoluble, so that their ionic strength in the concentrations used is lower than that of normal saline. The aldehydes, which do not ionize, cannot be used at high concentrations because denaturation of the protein proceeds too rapidly and tissue distortion results. On the other hand, at low concentrations (5 or 10 per cent) they are hypotonic. We have used the freezing-point-depression method⁽¹⁾ to determine ionic strengths of the various fixatives and then have added required amounts of salts to arrive at the proper tonicity.

The material used throughout to test fixation was grasshopper testis. For this tissue, the ionic strength of the fixing solution is equivalent to 0.9% NaCl. Such a solution will hereafter be referred to as normal saline equivalent (NSE). However, normal saline for other tissues may differ in salt concentration. The ionic strength of normal saline for a given tissue must be established and the fixing solution adjusted accordingly.

We have investigated the effects upon the tissue of various supplementary salts such as NaCl, KCl, MgCl₂ and CaCl₂, used singly as well as in balanced [e.g., Belar⁽³⁾] saline solutions. The fixing agents used in this phase of the investigation were OsO₄ and KMnO₄. When NaCl was used as the supplementary salt, the tissue matrix was finely granular. However,

under normal physiological conditions, potassium, rather than sodium, is found in cytoplasm. When KCl was used in the fixative the cellular contents appeared coarsely granular. This may or may not be normal; in any case the effect is decidedly different from that produced by the NaCl-supplemented solution. $MgCl_2$ and $CaCl_2$ enhanced the demarcation of membranes. On the other hand, a balanced saline supplement produced a smooth cell outline with relatively distinct membranes. We have concluded that enhancement or repression of structures within the cell can be a function of the salt used in the fixative and that this should be considered very carefully in the evaluation of electron micrographs.

Unbuffered fixatives adjusted to normal saline tonicity usually have an acid pH. The commonly-used osmium fixative is buffered to pH 7.4 with veronal-sodium acetate, a buffer never found normally in cells. Luft⁽⁴⁾ recently reported that unnatural buffers such as veronal and arsenate remove a considerable amount of protein from the tissues during fixation; this was shown by labeling proteins with C^{14} and demonstrating that some of the labeled material was leached out during fixation. He found that fixatives buffered with phosphate, a normal component of cells, extracted the least protein during fixation. In our experiments NSE fixatives were buffered with phosphate at pH 5.8 to 7.3. From preliminary work, it appears that as the isoelectric point of proteins is approached more granularity is seen throughout the cell. Since the isoelectric points of various proteins differ, it will be essential in the future to use fixing solutions of different pH values to obtain specific information. We have noted the breakdown of the nebenkern or the disappearance of centrioles in spermatid material when pH was altered. It is also known that mitochondria tend to disappear in acid fixatives.⁽⁵⁾ We expect to pursue the problem of pH specificity in greater detail.

Figures 40 and 41 are electron micrographs of grasshopper spermatocytes fixed in $KMnO_4$ at two different pH's. Note that in the tissue fixed at pH 6 (Figure 40) the nucleus has distinct areas of granulation which provide greater chromatin detail. At pH 7.3 (Figure 41) the nucleus is almost entirely granular with little, if any, chromatin distinction. The Golgi apparatus appears distinctly different at the two pH's. At pH 7.3 the vacuolar structure associated with the apparatus is noticeable, whereas at pH 6 the lamellar portion is well outlined and the vacuolar portion is less distinct. Differences are also seen in the endoplasmic reticulum, which has a distinct membrane and an absence of ribosomes at pH 6, while at pH 7.3 the membrane is less clear and ribosomes are visible. Mitochondrial membranes appear single at pH 7.3 but are definitely double at pH 6. The density of specific structures in the intercellular material differs at the two pH's. The best results to date on grasshopper testis material have

Figure 40. Grasshopper spermatocyte fixed in NSE , KMnO_4 , phosphate buffer pH 6.0. Lead citrate stained. Within the nucleus the chromatin material is distinct and granules of two sizes can be seen in the karyoplasm. The membranous components of the cell are distinct and are enhanced by the fine textured matrix of the cytoplasm. The endoplasmic reticulum appears smooth and does not show ribosome attachment. The Golgi apparatus appears to be mainly lamellar with a few indistinct vacuoles.

Figure 41.

Grasshopper spermatocyte fixed in NSE , KMnO_4 , phosphate buffer, pH 7.3. Uranyl acetate stained. Within the nucleus the chromatin masses are almost indistinguishable from the remainder of the karyoplasm. The cytoplasmic matrix is granular and the membranous structures appear coarse, although ribosomes are visible along the endoplasmic reticulum. The Golgi apparatus appears to be mainly vacuolar with only a slight indistinct membranous portion.

IS - Intercellular space M - Mitochondrion NU - Nucleus CH - Chromosome GO - Golgi apparatus ER - Endoplasmic reticulum

been obtained with fixative of the following composition:

KMnO₄: 0.13 g

Buffer (pH 5.9): 1.20 ml

M/15 KH₂PO₄, 9 ml;

M/15 Na₂HPO₄, 1 ml;

9% NaCl, 10 ml

H₂O, to make 10.0 ml

Final pH: 6

We will next attempt to use manganese and aluminum salts in the fixing solution, because these salts are catalysts for certain enzymes applied to tissue sections in cytochemical studies.

We feel it may someday be possible to define the fixation capacity of a given solution by virtue of all of its constituents and their concentrations. Such specificity of fixing and staining procedures would greatly increase the usefulness of electron microscopy for biological materials.

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