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PROGRESS REPORT: RADIATION AND IMMUNITY IN MICE INFECTED WITH TRYPANOSOMA DUTTONI

India Ink Blockade

Bernard N. Jaroslow and Frank A. Fraembs

Previous results⁽¹⁾ have shown that increased parasitemia and enhanced reproductive activity of Trypanosoma duttoni, a hemoflagellate of the mouse, follows whole-body irradiation of the host with 550 r. In irradiated infected mice the incidence of division forms increases from a near normal level at the start of the infection to a peak twice that found in non-irradiated infected mice. In mice that have received a course of India ink injections, this infection is characterized initially by a markedly higher percentage of division forms than that in the irradiated mice or the non-irradiated controls (Figure 1). Blockade plus irradiation appears to exhibit a synergistic effect, according to these preliminary results. The parasitemia, however, is unaffected by blockade, and the development of acquired immunity in the blocked, non-irradiated animals is temporally and quantitatively identical with that of the non-irradiated controls. The seeming divergence in the curves for parasitemia between irradiated and blocked, irradiated mice occurs after the eighth day when most of the animals have died; consequently the means are based on a very small sample of the group.

Figure 1. Effects of India ink blockade on infected mice

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We suggest that the macrophages play an important role in controlling the numbers of division forms, possibly by preferentially phagocytizing the less agile dividing forms. India ink blockade lowers the phagocytic activity of the macrophages; the phagocytic removal of cellular debris after irradiation probably has the same effect upon the efficiency of this process, resulting in a later increase in the occurrence of division forms of T. duttoni.

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1. Jaroslow, B. N. Effects of irradiation on innate and acquired immunity. Quarterly Report of Biological and Medical Research Division, Argonne National Laboratory. ANL-5247, p. 51 (1954).

HEMOLYSIN PRODUCTION IN IRRADIATED RABBITS. PART VI

William H. Taliaferro* and Bernard N. Jaroslow

It was reported previously⁽¹⁾ that in rabbits preirradiated with 400 r of X-ray antibody production was increased to near normal levels when yeast autolysate was mixed with sheep red cell antigen prior to injection. Preliminary studies on the restoration principle in yeast autolysate suggest that the factor(s) is (are) incompletely inactivated by exposure of yeast extract to 90°C in a water bath for 10 min or by dialysis against tap water for 15 hr, or by a combination of both treatments. Injection of a mixture of antigen and ribonuclease, a heat-stable protein, into irradiated rabbits resulted in a significant elevation in the amount of antibody produced. The data are presented in Table 1.

TABLE 1

Effects of heated and/or dialyzed yeast autolysate or ribonuclease on hemolysin production in irradiated rabbits

Group	No. of rabbits	Mean log peak titer	Geometric mean
Unirradiated	38	3.225 $\pm$.02	1700
400 r + yeast autolysate	25	3.125 $\pm$.03	1333
400 r + heated yeast	7	2.750 $\pm$.13	560
400 r + dialyzed yeast	7	2.850 $\pm$.37	710
400 r + heated dialyzed yeast	8	3.000 $\pm$.18	1000
400 r + ribonuclease	10	2.875 $\pm$.16	750
400 r	42	2.375 $\pm$.13	240

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*Department of Microbiology, University of Chicago.

PROGRESS REPORT: DISEASES OF LABORATORY ANIMALS

Acariasis of Mice

Robert J. Flynn

As a continuation of the study of acariasis in mice, (1, 2) the toxicity and acaricidal effectiveness of Aramite-15W* have been further investigated.

Toxicity. To determine whether Aramite treatment of mice would interfere with the results of subsequent radiation experiments, a shipment of 196 CF No. 1 female mice was divided into Aramite-treated and untreated, and X-irradiated and unirradiated groups as shown in Table 2. The treated mice were dipped twice, at 5-6 and 6-7 weeks of age, in a 2% suspension of Aramite-15W (with Nacconol** 1:1000). The irradiations were carried out when the mice were 8-9 weeks of age.

The 30-day LD₅₀ of the Aramite-treated mice (596.75 ± 10.20 r) did not differ significantly from that of the untreated mice (587.58 ± 8.18 r).

Effectiveness. The comparative effectiveness of single and repeated Aramite dippings is shown in Table 3. In this experiment, CF No. 1 female mice 13 months old, (10 per cage) were dipped once, twice, or three times, at weekly intervals, in a 2% Aramite-15W solution. Three months later, one mouse from each cage was examined for mites. Although one dipping will usually completely free a mouse of these ectoparasites, a few mites or eggs may survive; therefore two or more applications are necessary for complete eradication.

The mice of all treated groups which are now 21 months old are very similar in appearance (Figure 2). There are no epilated, ulcerated, or lacerated areas. The skin is pink, and the hair coat is full, white, and very soft to the touch. The untreated mice (Figures 3 and 4) are strikingly different. They show large areas of self-inflicted lacerations, the skin is whiter in color than that of the treated animals, and the coat is sparse, greyish-white, and coarse to the touch.

During the 8 months that these mice have been observed since they were treated, 23 of the original 37 mice receiving multiple dippings have died (62.2%) compared with 45 of the original 56 that were not dipped (80.3%).

*Active ingredient 2-(p-tert-butylphenoxy) isopropyl-2-chloroethyl sulphite. Naugatuck Chemical Co.

**Wetting agent manufactured by National Aniline Division, Allied Chemical and Dye Corp.

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TABLE 2

Effect of Aramite treatment on X-ray toxicity

Cage No.	Treatment	Dosage (r)	Number of mice	Survivors (30 days)
1	Dipped	525	9	7
2	-	525	9	8
3	Dipped	550	10	9
4	-	550	10	7
5	Dipped	575	10	4
6	-	575	10	8
7	Dipped	575	10	8
8	-	575	10	7
9	Dipped	600	10	6
10	-	600	10	5
11	Dipped	600	10	4
12	-	600	10	2
13	Dipped	625	10	4
14	-	625	10	4
15	Dipped	650	9	1
16	-	650	9	0
17	Dipped	-	10	10
18	-	-	10	10
19	Dipped	-	10	10
20	-	-	10	10

Treated LD50/30 = 596.75 ± 10.1975 r. Untreated LD50/30 = 587.575 ± 8.1825 r.

TABLE 3

Effectiveness of repeated dippings with Aramite-15W

Cage No.	Number of dippings	<u>Myocoptes musculus</u>	<u>Myocoptes romboutsii</u>	<u>Myobia muscili</u>	<u>Radfordia affinis</u>
1	3	-	-	-	-
2	3	-	-	-	-
3	2	-	-	-	-
4	2	-	-	-	-
5	1	+(Few)	-	-	-
6	1	-	-	-	-
7	1	+(Few)	-	-	-
8	None	+	+	+	-
9	None	+	+	+	-
10	None	+	-	+	+
11	None	+	+	+	-
12	None	+	+	+	+
13	None	+	+	+	-

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Figure 2. Treated 18-month-old CF No. 1 female mouse.

Figure 3. Untreated 18-month-old CF No. 1 female mouse.

Figure 4. Untreated 18-month-old CF No. 1 female mouse.

Figure 5. C57 Black female mouse before isolation.

Figure 6. C57 Black female mouse after isolation.

Figure 7. CF No. 1 female mouse before isolation.

Even though the pathology associated with each specific ectoparasitic infestation has not been clearly defined, this experiment shows that mice free of ectoparasites have decidedly better hair coats, fewer skin lesions, and apparently a longer life span than infested mice.

Nonparasitic Skin Disorders

The elimination of the external parasites has simplified the definition and study of nonparasitic skin disorders of mice.

The epilation over the back, frequently observed among C57 blacks (Figure 5), did not respond to acaricidal therapy. The skin appeared normal; the hairs were normal in number but broken off near the skin. When the animals were caged individually, the coat returned to normal within 7-14 days (Figure 6); when regrouped in pairs, the individual "hair-chewers" were identified.

Absence of all hairs of the nose included the vibrissae was frequently observed among CF No. 1 female mice (Figure 7) and was previously thought to be caused by the myobiid mites; however, the condition did not respond to acaricidal therapy. The skin appeared normal; the hairs were not broken off above the skin, but were either plucked out or completely absent. When these mice were caged individually, the hairs returned and grew back to normal length in about 30 days; when regrouped in pairs, the individual "whisker-eaters" were identified.

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1. Flynn, R. J. Progress report: Acariasis of mice. Quarterly Report of Biological and Medical Research Division, Argonne National Laboratory. ANL-5486, pp. 9-12 (1955).
2. Flynn, R. J. Progress report: Diseases of laboratory animals. Acariasis of mice. Report on Biological, Medical and Biophysics Program, Argonne National Laboratory. ANL-5518, pp. 97-99 (1956).

THE ANALYSIS OF OBSERVATIONS WITH DIFFERING WEIGHTS

Joan M. Gurian

The Analysis of Variance is used to estimate components of variance, according to a mathematical model which separates the several effects and interactions. If all observations have equal weight, the estimation of the mean squared-deviations used for this is the same as that used for hypothesis testing. However if the weights differ, the mean squared-deviations will be over- or underestimated, depending on whether the average weight is larger or smaller than one. This can easily be seen by looking at the analysis of a one-way classification, with k cells, each containing n items.

Let x_{ij} be the i th observation in cell j , $\bar{x}_{.j}$ the mean of the observations in cell j , and $\bar{x}_{..}$ the mean of all observations. The analysis is as follows (SS is the sum of squared deviations, df the degrees of freedom, and MS the mean squared-deviations):

Source of Variation:	SS	df	MS	Expectation of MS
Total	$\sum_{ij} (x_{ij} - \bar{x}_{..})^2$	$nk - 1$	$\frac{\sum_{ij} (x_{ij} - \bar{x}_{..})^2}{nk - 1}$	
Between cells	$n \sum_j (\bar{x}_{.j} - \bar{x}_{..})^2$	$k - 1$	$\frac{n \sum_j (\bar{x}_{.j} - \bar{x}_{..})^2}{k - 1}$	$\sigma_W^2 + n\sigma_B^2$
Within cells	$\sum_{ij} (x_{ij} - \bar{x}_{.j})^2$	$k(n - 1)$	$\frac{\sum_{ij} (x_{ij} - \bar{x}_{.j})^2}{k(n - 1)}$	σ_W^2

If the observations have weights w_{ij} the analysis takes the following form:

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Source of Variation:	SS	df	MS	Expectation of MS
Total	$\sum_{ij} w_{ij} (x_{ij} - x_{..})^2$	$nk - 1$	$\frac{\sum_{ij} w_{ij} (x_{ij} - x_{..})^2}{nk - 1}$	
Between cells	$\sum_j \left[(x_{.j} - x_{..})^2 \sum_i w_{ij} \right]$	$k - 1$	$\frac{\sum_j \left[(x_{.j} - x_{..})^2 \sum_i w_{ij} \right]}{k - 1}$	$w \left[\sigma_W^2 + n\sigma_B^2 \right]$
Within cells	$\sum_{ij} w_{ij} (x_{ij} - x_{.j})^2$	$k(n - 1)$	$\frac{\sum_{ij} w_{ij} (x_{ij} - x_{.j})^2}{k(n - 1)}$	$w \sigma_W^2$

The factors w_{ij} enter into all the sums. If we set all w_{ij} equal to w , the MS column can be rewritten:

Source of Variation:	MS
Total	$\frac{w \sum_{ij} (x_{ij} - x_{..})^2}{nk - 1}$
Between cells	$\frac{wn \sum_{ij} (x_{.j} - x_{..})^2}{k - 1}$
Within cells	$\frac{w \sum_{ij} (x_{ij} - x_{.j})^2}{k(n - 1)}$

Each sum is multiplied by a factor w , which must be removed to obtain proper estimates of the various components. A good estimate of this factor is the harmonic mean of all the w_{ij} . In testing hypotheses by the variance-ratio test, this factor is cancelled, so the ratio is not affected.

The same correction must be applied in the regression of weighted observations. To obtain an estimate of the residual variance, which is the mean variance of a point y , after the variance due to differing x 's has been removed, the SS must be divided not only by the degrees of freedom, but also by w , the harmonic mean of the w_{ij} , so that the residual variance has the following form:

$$V_{y.x} = \frac{\sum_i w_i (y_i - \bar{y})^2 - \frac{\sum_i w_i (x_i - \bar{x}) (y_i - \bar{y})}{\sum_i w_i (x_i - \bar{x})^2}}{w (n - 2)}$$

where x_i , y_i are the independent and dependent variable respectively, with weight w_i .

An example of the application of the correction for weights follows. The data are the 30-day survivals of mice exposed to single doses of X-ray; five tests were made, at two doses each. It was of interest to determine the components of variance due to the different tests. The variable is the arcsine transform of the percentage survivals. The weights are the total number of mice exposed at each point; they range from 11 to 16, with a harmonic mean of 13.12.

Source of Variation:	SS	df	MS	MS/w	Expectation
Total	10824.19	9	1202.68		
Between cells	8706.41	4	2176.60	165.90	$\sigma_W^2 + 2\sigma_B^2$
Within cells	2117.78	5	423.56	32.28	σ_W^2

Component of variance within cells: σ_W^2 : 32.28

Component of variance between cells: σ_B^2 : 66.81

Taking the square root of the within-variance-component to get an estimate of the standard deviation within cells, it is obvious that the MS before division by w is too large, since that gives a σ_W of 20.58, yet the largest deviation from a cell mean is 6.2. If we take the square root of the corrected MS we obtain $\sigma_W = 5.68$, a value which is larger than 70% of the deviations from the cell mean. Since one standard deviation includes 65% of all values, this is a good approximation.

I wish to express my gratitude to Dr. Douglas Grahn for suggesting the problem and furnishing the data used in the example.

EMBEDDING AID FOR COVERSIP MOUNTS IN ELECTRON MICROSCOPY

B. R. Nebel and O. T. Minick

Borysko and Sapranaukas⁽¹⁾ and independently Gay⁽²⁾ have described a method of making coverslip preparations of biological material from which small selected cell groups or single cells may be transferred to methacrylate for sectioning for electron microscopy. In both procedures it is suggested that the selected material viewed under light and phase microscopy be covered with a capsule containing partially polymerized methacrylate for final polymerization and hardening. The essential aim is to place a capsule filled to the brim with partially polymerized methacrylate open end down on the selected cell or cell group, so that the latter can be in the center of the (future) block. The present authors found it difficult accurately to invert and position the capsule with reference to a single cell or a small group of cells in a single cell layer. Such positioning is readily accomplished by the use of the auxiliary equipment described here, which can be built at low cost (about 4 shop hours).

Figure 8 shows the apparatus used. The insert shows a Lucite block (LBL) containing a central canal drilled to hold loosely a capsule filled to the brim with partially polymerized homogeneous methacrylate. The capsule is held by the setscrew (SSC) so that the open end projects about 0.15 mm above the surface of the Lucite block. Block and capsule are now placed on the stage of a light microscope with the condenser removed. The coverglass mount,^(1,2) in which the methacrylate is also partially polymerized, is inverted and observed in contact with the methacrylate in the capsule under the light microscope. It is now easy to arrange the essential material centrally over the surface area of the prospective block by gently shifting the cover glass.

This done, the coverglass may be "tacked" to the capsule in the desired position by running a small amount of partially polymerized methacrylate under the coverglass. The inversion can now be executed without hesitation; surface tension will hold the coverglass in place, while the entire assembly shown in the insert of Figure 8 is turned upside down by hand. Next the coverglass preparation with the attached Lucite block is placed coverglass down on a slide which rests on the baseplate (BPL). The setscrew (SSC) of the Lucite block is loosened, and the ramrod (RR) is positioned and lowered until its cupped end engages the closed end of the capsule. After the setscrew (SSC₁) is tightened, the preparation may be considered secure, and finally the Lucite block may be elevated out of reach of the capsule (CPS in the figure).

Figure 8. Assembly of apparatus. Lucite block (LBL), base plate (BPL), ramrod (RR), capsule (CPS), setscrews (SSC) and (SSC₁). The insert shows the Lucite block and setscrew in greater detail.

The ramrod is then lifted, freeing the capsule, and the base slide, coverslip, and capsule are removed to an oven for final polymerization.

After 48 hr at 60°C the coverglass may be removed from the methacrylate surface with a razor blade, after brief cooling of the coverglass and after placing a drop of acetone at the periphery of the open end of the capsule. After proper trimming of the final block no difficulty is experienced in finding the selected cells for sectioning.

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2. Gay, H. Serial sections of smears for electron microscopy. Stain Technol. 30: 239-242 (1955).

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AN ESTIMATE OF THE TOTAL NUMBER OF MOLECULES SUBJECT TO TRANSMUTATION WHEN THE NUMBER IS SMALL

Sylvanus A. Tyler

In the previous quarterly report⁽¹⁾ it was shown that, given pairs of values (i, T_i) $i = 0, 1, 2, \dots, p$, $p \leq N$ where T_i is the interval of time between the i th and the $i + 1$ st disintegrations, the estimate of the decay rate (k) and its associated standard error given by least squares are

$$\hat{k} = \frac{|\lambda_3|}{|\lambda_1|} ; \quad \hat{\sigma}_k = \left[\frac{\hat{\sigma}^2 \sum w_i}{|\lambda_1|} \right]^{1/2} \quad (1)$$

The quantity $|\lambda_j|$ is the determinant of the minor obtained by deleting the j th column of the matrix

$$\lambda = \begin{pmatrix} \sum \frac{w_i}{T_i} & \sum w_i & \sum w_i i \\ \sum \frac{w_i i}{T_i} & \sum w_i i & \sum w_i i^2 \end{pmatrix} \quad (2)$$

The appropriate weighting factor for T_i was found to be $w_i = 1/(N^1 - 1)^2$ where N^1 is an a priori approximation of the number (N) of molecules untransformed when observations begin. In like manner, the least squares estimate and standard error of Nk under the derived model

$$\frac{1}{T_i} = Nk - ki \quad (3)$$

are

$$\hat{Nk} = \frac{|\lambda_2|}{|\lambda_1|} ; \quad \hat{\sigma}_{Nk}^2 = \frac{\hat{\sigma}^2 \sum w_i i^2}{|\lambda_1|} \quad (4)$$

Since the estimates of N and k are independent random variables, $\hat{N}$ and $\hat{\sigma}_N^2$ are approximately given by

$$\hat{N} = \frac{\hat{Nk}}{\hat{k}} \left[1 + \frac{\hat{\sigma}^2 \sum w}{\hat{k}^2 |\lambda_1|} \right]$$

$$\hat{\sigma}_N^2 = \left[\frac{(\hat{Nk})^2}{\hat{k}^2} \right] \left[\frac{\hat{\sigma}_k^2}{\hat{k}^2} + \frac{\hat{\sigma}_{Nk}^2}{(\hat{Nk})^2} \right] \quad (5)$$

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It has tacitly been assumed in this formulation that the events (i) occur as a result of the parent disintegration process only and that all i's are recorded. As a rule, extraneous events, such as counts due to background and/or daughter products appear and are indistinguishable from the events of interest. Present also is the problem of "geometry" which largely depends on the specifications of the equipment used. We shall continue to assume 100% geometry.

The presence of extraneous events can sometimes be suspected merely by an inspection of the ordering of interval lengths. From empirical studies, $\hat{N}$ proved to be an exceedingly robust estimate of the number of molecules involved in the parent decay process. If the process is followed to completion and N^1 is assumed to be the total number of events observed, a difference of even one between N^1 and $\hat{N}$ infers the existence of foreign events. A bias in N affects the estimate of k to such an extent that $\hat{k}$ will be far from the true k . Thus an adjustment of N^1 and the intervals T_i must be made subjectively, and the adjustment must be judged by the reduction achieved in the errors of $\hat{k}$ and $\hat{N}$.

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1. Tyler, S. A. On the determination of the decay rate when the substance subject to transmutation is small. Report on Biological, Medical and Biophysics Programs, Argonne National Laboratory. ANL-5518, pp. 116-118 (1956).

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A NEW VARIANT FOR T5 RESISTANCE IN ESCHERICHIA COLI

Herbert E. Kubitschek and Harold E. Bendigkeit

Caffeine induces two types of T5-resistant mutants in E. coli, strain B/1,t. One of these substrains, which we designate E, is well-known; it appears to be completely resistant to the bacteriophage T5, and, in the presence of about 10^{10} phage particles per ml, single resistant cells give rise to countable colonies with 24 hr at 37°C in nutrient agar. The new variant, designated L, does not give countable colonies with this concentration of T5 until at least two days have elapsed, but when T5 is absent colonies come into view as readily as with the E variant. The L variant has been found previously by A. Novick.⁽¹⁾

The addition of caffeine in concentrations of the order of 150 to 450 mg/l to tryptophan-limited cultures of B/1,t in the chemostat induces about twice as many L bacteria as E bacteria. The growth rates of the two strains appear indistinguishable from that of the parent culture. The characteristics of the L strain are under study.

Reference

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A CONSIDERATION OF HEMATOPOIETIC BALANCE

Harvey M. Patt

The relative constancy of the cellular components of blood presents one of the intriguing problems of homeostasis. Although a reasonably quantitative relationship between blood cell production and destruction may be inferred in the steady state, the correlating mechanisms are not well defined. This hiatus exists for all of the blood cells but is particularly obvious in the case of the leucocytes. It is generally recognized that the problem of regulation must be thought of in terms of several parameters, which is to say that it is necessary to consider the level of cells in transit against the background of production, utilization and storage. A direct approach to evaluation of marrow capacity and productivity is complicated by the diffuse distribution of the blood-forming sites and the possible variability of their components, e.g. relative amounts of active and inactive marrow. A reasonable approach to production may be made from the point of view of utilization in the case of erythrocytes, which are ordinarily confined to the circulation. This situation, however, does not obtain with the mature granulocytes which have a more uncertain peripheral distribution and termination.

An interesting theoretical consideration of the balance of hemic cells has been made by Osgood.⁽¹⁾ An erythrocyte production rate derived from life span measurements and a maturation time based on clinical observations were used to estimate the marrow erythrocytic component. The number of myeloid cells in marrow was then computed from the myeloid-erythroid ratio and the distribution of the various classes from observation of the life spans in tissue culture. It is the purpose of this paper to present an independent estimate of myeloid-erythroid balance, the fundamental difference being that the computation centers about the comparative mitotic activities of the two lineages rather than assumed maturation times.

Mitotic index and intermitotic time of myeloid and erythroid cells.

Studies of human marrow as reported by Japa⁽²⁾ reveal a mitotic index of 1.5 per cent with an essentially equal distribution (actually 45:55) of mitoses between myeloid and erythroid components. We may assume, as did Osgood,⁽¹⁾ a myeloid-erythroid ratio (M/E) of 3 in marrow uncontaminated with blood and also that only one-sixth of the myeloid and one-fourth of the erythroid cells are capable of division. These would appear to be reasonable assumptions on the basis of available information.⁽³⁾ It follows then that the true mitotic index (M.I.) for the myeloid series would be 6 and for the erythroid series 12.

$$M.I._{\text{myeloid}} = 0.75 \left(\frac{100}{1/6(75)} \right) = 6 \quad (1)$$

$$M.I._{\text{erythroid}} = 0.75 \left(\frac{100}{1/4 (25)} \right) = 12 \quad (2)$$

Since myeloid and erythroid cells eventually reach a stage of maturation beyond which cell division does not occur, production of cells in the normal steady state with constant population must be expressed arithmetically rather than exponentially. Hence, the intermitotic time is given simply by the ratio of the mitotic time to the mitotic index ($IT = MT/MI$). The mitotic time is believed to be reasonably constant for many cell types and is of the order of a half hour from the point of recognizable elongation of chromosomes.⁽⁴⁾ This stage is also generally the starting point for determination of the mitotic index. On this basis, the intermitotic time for the myeloid cells is estimated to be 8.3 hr and for the erythroid cells 4.2 hr.

Erythropoiesis. There are 27×10^{12} erythrocytes in circulation in standard man (5.4×10^9 cells per ml; blood volume, 5×10^3 ml). Since the average life span of the circulating cells is about 120 days, erythrocyte production must equal 225×10^9 cells per day or 9.4×10^9 per hr under normal physiological conditions. If the intermitotic time is 4.2 hr, there must be about 6 mitoses per day for each cell capable of division.

$$1/6 (225 \times 10^9) = 37.5 \times 10^9 \text{ cells in the generative pool} \quad (3)$$

If dividing cells represent one-fourth of the total, there will be 112.5×10^9 cells in the maturation pool, and the total number of nucleated cells will equal 150×10^9 . The maturation time, i.e., time to enucleation, is given by the ratio between the number of maturing cells and the number of cells, presumably reticulocytes, released per day.

$$\frac{112.5 \times 10^9}{225 \times 10^9} = 0.5 \text{ day or } 12 \text{ hr maturation time} \quad (4)$$

Granulopoiesis. We may recall the essentially equal distribution of mitoses in the myeloid and erythroid series.⁽²⁾ If the mitotic time is the same, the production of new cells may be assumed to be equivalent or 225×10^9 granulocytes per day in the physiological steady state. This means that the 25×10^9 granulocytes detectable in the circulation of standard man are replenished nine times a day and that the turnover time or length of a single sojourn in circulation is 2.7 hr. If the intermitotic time is 8.3 hr, there must be about 3 mitoses per day for each cell capable of division.

$$1/3 (225 \times 10^9) = 75 \times 10^9 \text{ cells in the generative pool} \quad (5)$$

If dividing cells represent one-sixth of the total, there will be 375×10^9 cells in the maturation pool and the total number of cells in marrow will equal 450×10^9 . The time spent in the marrow after division is equal to the ratio of the number of cells in maturation to the number of cells released per day.

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$$\frac{375 \times 10^9}{225 \times 10^9} = 1.7 \text{ days or } 40 \text{ hr maturation time} \quad (6)$$

These considerations present points of agreement and disagreement with the values reported previously.⁽¹⁾ The salient data are compared in Table 4. These calculations have in common the assumptions concerning erythrocyte production, myeloid-erythroid ratio and the fraction of cells capable of mitoses in the two situations. The fundamental difference is derived from the fact of an assumed maturation time in the one case⁽¹⁾ and the utilization of mitotic counts in the other. An erythrocyte maturation time of 48 hr is based mainly on the time of onset of reticulocytosis after iron therapy of iron-deficient anemic patients. This time is perhaps an overestimate, since it does not take into account various possibilities for a lag in incorporation or the sensitivity of the measurement in relation to the daily turnover. It is perhaps pertinent that a decrease in blood reticulocytes and in iron incorporation is manifest experimentally within a day after moderate doses of X-radiation.^(5,6) Since such dosages do not interfere with iron uptake by the nondividing erythroid cells,⁽⁷⁾ it may be concluded that the effect is related to an inhibition of new cell formation. From these data, it would appear that erythrocyte maturation time in experimental animals at least may be somewhat less than a day. It should be noted that the calculated erythroid maturation time in man refers only to the time required for enucleation, which may or may not be equivalent to the total time spent in the marrow after division. The granulocyte maturation time used by Osgood⁽¹⁾ is derived from observations in tissue culture; the present estimate is reasonably close to this value. The data of Ottesen⁽⁸⁾ and of Kline and Clifton⁽⁹⁾ on P³² incorporation of DNA of leucocytes indicate that labeled cells begin to appear in the circulation at about 4 days. This time is of the same order as the tissue culture and theoretical estimates of the time spent in the marrow (2 to 3 days), if we assume a period of maximal labeling of about 24 hr.

Mitotic counts are, of course, also subject to appreciable error. This, however, would probably be in the direction of an underestimate. The distribution of mitoses between myeloid and erythroid cells is a critical datum. The paucity of quantitative information along these lines in man is regrettable. It will be remembered that the assumption of a mitotic time of one hour rather than of one-half hour would increase the estimated values (Table 4), with the exception of the production rates, by a factor of two. Underestimation of the true mitotic time in the computation would be compensated for, in part at least, since a similar situation would probably also apply to the mitotic index.

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TABLE 4

Comparison of values for erythroid and myeloid components
of human marrow

Index	Erythroid		Myeloid	
	Present calc.	Osgood(1)	Present calc.	Osgood(1)
Total nucleated cells $\times 10^{-9}$	150	600	450	1800
Generative cells $\times 10^{-9}$	37.5	150	75	300
Maturing cells $\times 10^{-9}$	112.5	450	375	1500
Intermitotic time, hr	4.2	16	8.3	12
Maturation time, hr	12	48	40	60
Cells formed per day $\times 10^{-9}$	225	225	225	600

Although the values derived from mitotic counts indicate an equivalent production of erythrocytes and granulocytes, the presumed functional capacity of actively dividing myeloid elements is about twice that of the erythroid. In view of the respective roles and life spans of the descendants of the two series, this factor of safety is not an unreasonable one. It is perhaps noteworthy that erythrocytic precursors are believed to be more radiosensitive than the granulocytic by histologic criteria.⁽¹⁰⁾ Studies of Fe^{59} uptake by rat erythrocytes reveal a significant inhibition of erythropoiesis 24 hr after exposure to doses as low as 25 r.⁽⁶⁾ The apparent difference in radiosensitivity is consistent with the estimated shorter intermitotic time of erythroid cells.

The present computations confirm the conclusion of Osgood⁽¹⁾ that only a small proportion of the granulocytes outside of marrow is detectable in the circulating blood. If we accept a peripheral life span of 8 to 10 days from the studies of Ottesen⁽⁸⁾ and of Kline and Clifton,⁽⁹⁾ it would appear that there are some 75 times as many cells in the periphery as can be found in the blood by conventional counting and sampling methods and about 5 times as many in the periphery as in the marrow. There are already indications of the availability of large reserves of developing neutrophils in marrow relative to the number detectable in blood.^(11,12) A similar conclusion emerges from our experiments in which blood from a leucopenic X-irradiated donor is shunted through the isolated extremities of a normal dog. These experiments will be reported separately.

Consideration of the temporal course of the blood level of DNA-labeled cells and the estimated rate of granulocyte production would appear to lend credence to the suggestion by White⁽¹³⁾ that granulocytes may have many intravascular sojourns, each of which may be only a fraction of the total life of the cell. Since, however, there is an indeterminate number of granulocytes in vascular reservoirs and even in the effective circulation, differentiation of freely circulating cells from the total within the vascular bed is germane to such interpretation.

The values for hematopoietic activity presented in this paper are intended only as an approximation to the physiological picture, which may, of course, be quite different. In addition to the validity of the several assumptions and the reliability of the data upon which the computation depends, it may be noted, for example, that provision has not been made for the possible attrition of cells in the marrow. These estimates, however, provide a basis for further experimentation.

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THE METHYLATION OF HOMOCYSTEINE TO METHIONINE IN BACTERIA

Stanley K. Shapiro

It was reported previously^(1,2) that the following reaction occurs in normal and in ultra-violet induced methionine mutants of Aerobacter aerogenes: homocysteine + S-methylmethionine $\rightarrow$ 2 methionine. Cell-free extracts of A. aerogenes can carry out this reaction to 90-95% completion in three hours. The pH optimum of the reaction is wide, between 7.0 and 7.5. Of several strains of Escherichia coli that were tested for this reaction, only E. coli K₁₂ gave an active extract, but it seems unlikely that such an important reaction would not occur in other strains of E. coli. It would be more reasonable to suspect that the failure to detect the reaction depends on some other factor involved in the release of the enzymes from the cells. This will be investigated further.

In order to obtain more evidence for the methylation of homocysteine to methionine, C¹⁴H₃-labeled and S³⁵-labeled methylmethionine were synthesized and used as substrates for the enzyme system. The results are shown in Table 5. The observed transfer of C¹⁴H₃ from S-methylmethionine to methionine was as expected. This, however, would occur whether or not homocysteine was methylated. However, with S³⁵-methylmethionine as substrate, the specific activity of the resultant methionine was half that of the S³⁵-methylmethionine since half the sulfur must originate from unlabeled homocysteine.

TABLE 5

The transfer of C¹⁴H₃ and S³⁵ from S-methylmethionine to methionine by cell-free extracts of A. aerogenes¹

Substrate	Counts/min./ micromole	Counts/min/ micromole of isolated methionine	Per cent transfer
C ¹⁴ H ₃ - methylmethionine	36,236	19,686	52.9
S ³⁵ - methylmethionine	76,347	36,105	47.3

¹ Extracts were incubated with 2 μ M per ml of homocysteine and S-methylmethionine for 3 hours at 35°. Methionine was isolated from the reaction mixture by paper chromatography and eluted from the papers with 0.1% HCl. The concentration of the methionine was determined with the nitroprusside test and the radioactivity was measured in a gas flow chamber.

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This enzyme system has been extracted from cells of A. aerogenes grown in several media, including media containing methionine. It appears that this enzyme is normally present in those cells. Methyl donors that are active in other systems are all inactive in this system. S-adenosyl + methionine shows some activity as a methyl donor. S-methylmethionine has not been isolated as yet from A. aerogenes. However, since the compound is so active as a methyl donor in this system, it seems likely that a compound closely related to, if not identical with S-methylmethionine, is a normal methyl donor in the transmethylation reactions of bacterial cells.

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LIGHT AND ELECTRON MICROSCOPE STUDIES OF MORPHOLOGICAL
CHANGES OF MITOCHONDRIA DURING SPERMATOGENESIS
IN THE GRASSHOPPER

Theodore N. Tahmisian, E. L. Powers, and Rosemarie L. Devine

In grasshopper spermatogenesis the mitochondria undergo morphological changes. A comparative study of living and fixed material as seen with light and electron microscope indicates that the following steps take place. First, mitochondria migrate onto the meiotic spindles where they grow and divide equally by central constriction at the plane of cleavage. They elongate and become filamentous and the filaments form a crescent at a restricted plane about the nucleus. Nodes then appear at either end of this crescent, and the filaments become entwined at the nodes as the latter move toward each other. The newly formed nebenkern are derived from these nodes; they are fibrillar, but their fibrillar character undergoes dissolution; peripheral pockets are formed which coalesce until only two discrete bodies remain. Finally, the two bodies come into contact with the centrosome and occupy part of the space on either side of the tail filaments and sheath of the spermatozoon.

PROGRESS REPORT: DENSITY GRADIENT CENTRIFUGATION

Correction for Osmotic Pressure Changes in Estimation of Particle Size

John F. Thomson and Florence J. Klipfel

Recent work by Tedeschi and Harris⁽¹⁾ on osmotic effects in mitochondria has enabled us to take into consideration a factor which had previously given rise to uncertainty in the interpretation of data on enzyme distribution in cytoplasmic particulates isolated by density-gradient centrifugation.⁽²⁾ Although we had estimated the density of mitochondria of rat liver to be 1.22 g/cc, by means of equilibrium sedimentation in a sucrose gradient, we recognized that this figure was valid only for mitochondria in sucrose solutions of the same density (about 1.7 M). In less concentrated sucrose solutions (i.e., 0.25 M, the concentration used for homogenization of the tissue) the density of mitochondria should be appreciably less than 1.22 g/cc.

Tedeschi and Harris measured the sizes of mitochondria in varying concentrations of sucrose and then calculated the osmotic dead space and the relationships between size and sucrose concentration. We have incorporated these relationships into our formula for estimation of particle size,⁽²⁾ making the following assumptions: (a) at any given concentration of sucrose, the volume of the dead space is directly proportional to the total mitochondrial volume for all sizes of mitochondria; (b) at sedimentation equilibrium the density of the dead space is equal to that of the osmotically active space, and both are equal to that of the suspending medium; (c) the time required for the mitochondria to equilibrate to the progressively increasing osmotic gradient is sufficiently short to be considered negligible in relation to the total centrifugation time; and (d) the density of the osmotically active space parallels the density of the suspending medium at all sucrose concentrations employed.

We have thus incorporated into the formula two additional empirical expressions relating particle diameter and particle density to tube length. When this revised equation is used to construct particle size distribution curves, a value of 0.74 μ is obtained as the diameter associated with maximum succinic dehydrogenase activity (i.e., mitochondria); in view of morphologic considerations this is a more realistic figure than the value of 0.45 μ previously reported.⁽²⁾

There is the additional assumption inherent in this application, however, that all cytoplasmic particles are osmotically alike. Kuff and Schneider⁽³⁾ have shown that the osmotic behavior of uricase-containing particles is different from that of mitochondria. However, since it is virtually impossible to separate the former as a reasonably homogeneous

unit for study of their physical properties, we feel more justified in assuming the same behavior for all types of particles than in either making no correction for osmotic effects or choosing arbitrary correction factors with no experimental basis.

A Note on the Use of a Tissue Press Prior to
Homogenization of Rat Liver

In an earlier quarterly report⁽⁴⁾ it was mentioned that the mitochondria of mouse liver seemed to be larger than those of rat liver. We have subsequently established that this difference was referable to the use of a stainless steel tissue masher in the studies with rats. The results of a direct comparison between mashed and unmashed rat liver are shown in Figure 9, which indicates that when the tissue was mashed, a higher proportion of the cytochrome oxidase activity was found associated with smaller particles, and that the size associated with maximum activity was about 20% lower.

Figure 9. Effect of mashing prior to homogenization on the distribution of cytochrome oxidase in rat liver. Solid line, mashed; dotted line, not mashed.

Additional studies on distribution of succinic dehydrogenase in 6 rat livers have shown that there was appreciably less variation in distribution patterns among individual animals when the masher was not employed. The average coefficient of variation for the 24 points used in establishing the distribution curve was 28.0 when the masher was used, 19.7 when it was not. The average coefficients of variation for uricase distribution were even more revealing: 30.2 when the masher was used, 14.4 when it was not. The shape of the uricase distribution curve was not materially altered.

It should be pointed out, however, that although avoidance of the use of the masher resulted in a greater uniformity in distribution patterns, the activities of the particles per unit nitrogen were higher when the masher was employed. For mashed liver, a maximum activity of $4200 \mu\text{l O}_2/\text{hr}/\text{mg N}$ was observed for the most active succinic-dehydrogenase-containing fraction, more than 6 times that of the homogenate, which for this series averaged $680 \mu\text{l O}_2/\text{hr}/\text{mg N}$.⁽⁵⁾ Without the masher, the maximum activity was only 2800, although the average for the homogenate activity was somewhat higher, 800.

It is thus evident that use of the masher probably damages some of the mitochondria, so that significant amounts of enzyme activity are found associated with smaller particles which should have been associated with larger ones. On the other hand, the mitochondria which remain are quite able to esterify inorganic phosphate.⁽⁶⁾ It would thus seem that, in view of the smaller size of mitochondria associated with maximum activity, the masher is useful in freeing mitochondria from extraneous nitrogenous material, which presumably adheres to mitochondria isolated without mashing and results in their being of larger size and lower activity.

We have fed carbon-tetrachloride to a few rats to see whether the changes in distribution previously reported⁽⁵⁾ could be repeated without mashing the liver, and were able to substantiate our earlier findings; actually, the smaller degree of variation among the controls made the changes appear even more striking.

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PROGRESS REPORT: STUDIES ON THE FINE STRUCTURE AND
REACTIONS OF CONNECTIVE TISSUE FIBERS

Observations on Fibrillogenesis in the Connective Tissue of the
Chick Embryo by the Aid of Silver Impregnation

F. Wassermann and Lucille Kubota

A technique has been developed which combines silver impregnation and ultrathin sectioning for the electron microscopic demonstration of fibrils in the connective tissue of the chick embryo. Electron micrographs provide evidence for the specificity and completeness of the silver impregnation technique. It has been shown that, in this particular tissue and at the stage of development represented by our material, the argyrophil fibers are enclosed in the ramified bodies of the fibroblasts.

EFFECT OF AGE, PARITY, AND LITTER SIZE ON THE ACUTE
LETHAL RESPONSE OF FOUR INBRED MOUSE STRAINS TO
WHOLE-BODY X-IRRADIATION

Douglas Grahn, Robert J. Schweisthal, and Katherine F. Hamilton

A previous report by Grahn and Hamilton⁽¹⁾ presented the basic differences, in terms of the $LD_{50/30}$ values and dosage-mortality slopes, in the radiation response of strains BALB/c, A, C3H_f/He and C57BL/6. The data from which these parameters were obtained have been analyzed further in order to investigate the degree to which several underlying variables may alter the mortality following single-dose, whole-body exposure to X-irradiation.

In distributing the mice across the doses being given in any particular replication or test run, the commitment of whole litters to a dose was avoided. It has been previously demonstrated by Kaplan and Brown,⁽²⁾ that a heterogeneity of response occurs as a result of the tendency of litter-mates to respond more uniformly than mice of different litters. If whole litters are committed to a dose, then differences between litters became confounded with dose effects. This difficulty was minimized by distributing litter-mates as equally as possible across all the doses in each test.

Because the litter is the unit of variation for age and other characteristics of the litter history, the mean value for any litter factor such as age was nearly constant for all dose groups in each test. Since the data for each strain are composed of the results of 15 to 20 independent tests, chance variation caused each test to be characterized by a mean age that differed somewhat from those of all other tests. A preliminary examination of the data indicated that parity and litter-size as well as age had some effect on the response in all strains. In general, ages in these data ranged from 60 to 110 days, parity from 1 to 10, and litter size from 2 to 12.

In order to establish the effect of these variables on mortality, the main body of data for each strain was broken down into four reasonably balanced subclasses under each variable, i. e., age, parity, and litter size. Effort was made to retain rough numerical equality among subclasses while attempting to maximize the total range between the midpoints of the subclass intervals. The resulting 4 x 4 x 4 tables generally had 50-55 cells represented. Within each cell, the weighted means of dose and response were estimated so that the response could be adjusted to a constant dose, in this instance, the strain LD_{50} value. It was assumed that errors from the adjustment process were self-cancelling, and a standard multi-variate analysis was executed.

In strains BALB/c, A, and C3H_f, the most nearly linear inter-relationships for all variables were obtained on a straight arithmetic scale. In the C57BL strain a log transform of the mortality percentages was used. By means of the derived multiple regression equations (see Table 7), the test mean mortality values were adjusted to a constant age, parity, and litter-size for all strains. Adjusted test LD₅₀ values were then obtained. Table 6 presents the results of the above analysis.

TABLE 6

Mean test LD_{50/30} values, standard deviations, and variances before and after adjustment to a constant age, parity and litter size for all strains

Strain	No. of tests	LD ₅₀ ± SD unadjusted	LD ₅₀ ± SD adjusted	Variance between tests		Per cent change
				Unadjusted	Adjusted	
BALB/c	17	503.1 ± 32.6 r	500.2 ± 28.5 r	1060.5	809.8	- 23.6
A	30*	552.1 ± 24.2 r	552.3 ± 24.2 r	585.3	584.0	- 0.2
C3H _f /He	19	593.3 ± 25.9 r	593.2 ± 27.1 r	670.4	735.3	+ 9.7
C57BL/6	16	629.3 ± 19.9 r	630.4 ± 16.4 r	395.4	267.5	- 32.3

*15 tests each from sublines A/Jax and A/He.

While the LD₅₀ values changed very little as a result of the adjustment, the between-test variances showed irregular changes. In strains BALB/c and C57BL, the removal of age, parity and litter-size effects reduced the variance by 24% and 32%, respectively. Strain A showed essentially no change, while a small increase occurred in the C3H_f mouse. The relative success of the adjustment process is principally a function of the removal of age effects on mortality. Inspection of Table 7 indicates that age is the only variable that has a consistent correlation with response. However, the magnitude of the partial regression on mortality of age shows the best rank-order correlation with the changes in variance. This regression coefficient describes the expected change in the full 30-day mortality that will occur for each change in day of age at irradiation. The estimate is based on the use of a constant dose and applies to the age interval in these data, 60-110 days.

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TABLE 7

Multiple regression equations and partial correlations of mortality
with age, parity and litter size

Strain		Strain means		
		Age(X_1)	Parity(X_2)	Litter size(X_3)
	Regression equations			
BALB/c	$Y = 115.99 - 0.990X_1 + 2.174X_2 + 1.825X_3$	85.2	3.60	5.85
A	$Y = 118.45 - 0.640X_1 - 1.191X_2 - 0.878X_3$	87.2	3.37	5.66
C3H _f /He	$Y = 73.04 - 0.246X_1 - 0.765X_2 + 0.135X_3$	84.1	3.78	6.74
C57BL/6	$\log Y = 2.473 - 0.010X_1 + 0.035X_2 - 0.009X_3$	85.3	3.38	7.18
	Partial correlations			
	$rY1.23$ $rY2.13$ $rY3.12$			
BALB/c	- .486 + .244 + .213			
A	- .508 - .144 - .101			
C3H _f /He	- .335 - .161 + .025			
C57BL/6	- .589 + .512 - .156			

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Figure 10 expresses this age-dependence of acute lethality in terms of estimated LD_{50} values at a constant parity and litter size for all strains. The data show that strain differences in the LD_{50} will vary quite extensively with variation in mean age at test. Because of the small range in age in these data (84 to 87 days), the strain LD_{50} values were not appreciably altered by the removal of age effects on mortality.

Figure 10. Dependence of $LD_{50/30}$ values on age at exposure for all strains at a constant parity and litter size of 3.5 and 6.3, respectively.

The effect of parity and litter size was not consistent from strain to strain. This analysis may not be most efficient for these variables since litter size generally shows a shallow curvilinear relation to parity. The use of a linear relationship, as in this analysis, reduces the precision of estimates regarding these two variables.

The observed curvilinear relation between mortality and age in the C57BL strain is consistent with the data of Abrams⁽³⁾ for the same strain. In his experiments, with ages of 30, 45, 60, and 90 days and a constant

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dose of 550 r, mortality dropped sharply from 90% at 30 days to 19% at 60 days, but decreased only to 13.5% at 90 days. LD₅₀ values calculated from these observations are not inconsistent with the data given in Figure 10 and strongly suggest a sharp linear decrease in LD₅₀ occurs as age decreases from 60 to 30 days.

In the other three strains there is a similar linear dependence of LD₅₀ on age, but no data are available from which to ascertain the nature of the curve prior to or beyond the given age interval. It is possible that all strains have the same basic age-dependence curve, and that comparable responses occur, but at different chronological age intervals in each strain.

Kaplan and Brown⁽²⁾ did not detect an effect of parity or litter size on mortality in strain C57BL. The present data are consistent with respect to litter size; however, parity does show a partial correlation with mortality of + 0.51.

For most practical experimental purposes, it is reasonable to conclude that variation in parity and litter size can be ignored. Age, however, should be subjected to close control, unless evidence is available to indicate the existence of a very shallow age-dependence curve in the age interval employed.

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FURTHER STUDIES ON THE EFFECTS OF X-RADIATION
ON THE MULTIPLICATION OF RICKETTSIA MOOSERI
IN EMBRYONATE EGGS

Donald Greiff, E. L. Powers, and Henry Pinkerton

The effect of X-rays on growth of rickettsiae in the embryonate egg has been investigated. An intensifying effect of X-radiation on rickettsial growth was observed, which may be attributed to alterations of the cells of the host that persist at least 8 days. The greatest enhancement occurred when moderately infected cells were irradiated. The experiments indicate that X-rays may neutralize or reverse changes in the host that are unfavorable to the growth of rickettsiae.

PROGRESS REPORT: GAMMA RAY TOXICITY PROGRAM

Cytological Evidence for Irradiation Damage in Lymphoid Tissue
of Irradiated Mice

Samuel Leshner, Marietta Miller, and George A. Sacher

It is well known that in mice the lymphoid system is damaged by exposure to even low doses of irradiation, and that the degree of damage is dependent on the size and rate of the dose. Most of the evidence in support of this statement is based on the tabulation of lymphocyte numbers in the circulating blood following single dose irradiation. In this study we are concerned with the drop in lymphocytes in the peripheral blood and the correlation of this drop with the appearance of "debris" in the lymphoid tissues.

LAF₁ mice were exposed to continuous γ -irradiation at the rate of 2.5 kr/day, and sacrificed at closely spaced time intervals. An analysis of lymphocyte numbers showed that they dropped rapidly after 2 hours, reaching an extremely low level at 36 hr (Table 8).

TABLE 8

Effect of continuous γ -irradiation on lymphocyte count in mice

Time from start of exposure (hr)	No. of mice	Lymphocytes/mm ³
Controls	8	4532
$\frac{1}{2}$	4	3232
1	4	3746
2	4	4665
4	4	1558
6	4	1231
8	4	631
12	4	551
24	4	31
36	4	6

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An examination of the spleens, thymuses, and Peyer's patches of these mice revealed large numbers of darkly staining (hematoxylin and eosin) spherical globules. Histochemically these globules also stained intensely with toluidine blue (Figure 11) buffered at pH 4.0 (McIlvaine's buffer). Their basophilia was not noticeably reduced after treatment with ribonuclease (Figure 12). The globules were also Feulgen-positive (Figure 13). After treatment with desoxyribonuclease a Feulgen-negative component remained which still, however, stained with toluidine blue. When sections were treated with periodic-acid-Schiff reagent, the globules were completely negative (Figure 14). At this point it can be said only that the globules contain both ribo- and desoxyribonucleoprotein. Using crude estimates of the number of globules per given area of tissue it was found that the number increased rapidly after 4 hr and reached a peak at 12 hr, after which it dropped off even more rapidly; none were found after 36 hr. These changes correlate very closely with the fall in lymphocyte count in the peripheral blood.

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Figure 11. Spherical globules in Peyer's patch of ileum, stained with toluidine blue, concentration 1/2000, pH 4.0.

Figure 12. Similar area from the same Peyer's patch stained with toluidine blue after digestion with ribonuclease (0.1%). Stainability of globules not noticeably affected.

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Figure 13. Adjacent area stained by the Feulgen reaction.
Globules intensely Feulgen-positive.

Figure 14. Sections from same general area stained by the periodic-
acid-Schiff-reaction and counter stained with hematoxylin.

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PROGRESS REPORT: GAMMA RAY TOXICITY PROGRAM

A Cytological Study of the Intestine of the Mouse after Continuous
Total-Body Gamma Irradiation at the Rate of 2.5 kr./Day

Samuel Leshner, Anthony R. Sallese, and Katherine F. Hamilton

The sensitivity of the intestinal mucosa to irradiation has been intensively studied since the early reports of Warren and Whipple.⁽¹⁾ Most of these studies followed single-dose, total-body X-irradiation and few indeed were concerned with the sequence of events which led up to the maximal damage they described. The reports of Bloom and Bloom,⁽²⁾ Tillotson and Warren,⁽³⁾ and Montagna and Wilson⁽⁴⁾ were the exceptions.

In this preliminary report we are concerned with (a) the time sequence of cytologically detectable injury from 1/2 hr to 120 hr of continuous exposure to γ -irradiation, (b) the behavior of nucleic acids and periodic-acid-Schiff-reactive substances during the course of exposure, and (c) regional differences in sensitivity between the three levels of the small intestine and the colon.

Method

The calculated daily dose delivered by the 1000-curie cobalt source was 2.5 kr. Mice were sacrificed at 1/2, 1, 2, 4, 6, 8, 12, 24, 48, 60, 72, 84, 96, 108, and 120 hr. Three levels of the small intestine, i.e., the duodenum just distal to the pyloric sphincter, the jejunum at the midpoint of the small intestine and the ileum just before it joins the caecum, and also one section of the colon approximately 10 mm distal to the caecum, were removed and fixed. The fixative was a 20:2:1 mixture of alcohol, buffered neutral formalin, and glacial acetic acid.

Preparation of Sections - Sections were cut at 5 μ and stained with Delafield's hematoxylin and eosin, or, for the basophilic substances, in toluidine blue buffered to pH 4.0 with McIlvaine's buffer. To identify this basophilic substance further, control sections were incubated for two hours at 37° C in 0.1% ribonuclease buffered (McIlvaine's) to pH 6.8. The basophilic material removed by RN-ase digestion was cytoplasmic for the most part, and was considered to be RNA. For the identification of DNA, sections were stained by the Feulgen reaction.

To show the submucosal connective tissue, sections were treated with Van Gieson's stain, which produces a bright red color with collagen fibers. The periodic-acid-Schiff reaction (both before and after diastase digestion) serves three purposes, in that it shows the fine reticulin network of the submucosal connective tissue, gives a vivid reaction with both intra- and extracellular mucus, and reacts with the cuticular border of the mucosal epithelium.

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Results

For the first 36 hr the mice appear physically normal although they begin to lose weight at 24 hr. By 48 hr their fur has become ruffled and many have diarrhea. Movement of food from the stomach stops between 72 and 84 hr, and the entire intestine becomes an empty tube lubricated with an excessive exudate of somewhat bloody mucus.

Histologically the small intestine of the mouse does not have well defined microscopic "landmarks" which can be used as criteria for differentiating between the three levels. Radiologically they show differences in sensitivity; i.e., in their response to irradiation. The first morphologically visible damage appears in the nuclei of the duodenal crypt cells. The mucosal epithelium, submucosal connective tissue, and muscularis mucosae do not show damage until much later. Sensitivity to irradiation is much higher in the duodenum and damage is not only more intense but shows up at an earlier period. There is little difference in sensitivity between the jejunum and ileum. The colon is apparently quite resistant and shows very little effects of even higher doses until a few hours before death.

The following descriptions trace the sequence of changes in the development of damage in the mucosal epithelium of the crypts and villi, the submucosal connective tissue and the muscle layers, in all three sections of the small intestine and the colon. Complete photomicrographic coverage could not be given to the numerous changes that occur nor to the various time levels studied; however, the accompanying figures indicate the extent of damage, since Figures 15, 17, and 19 are photographs of sections from intestines of mice exposed to 8 hr irradiation, and Figures 16, 18, and 20 illustrate changes after 120 hr.

Mitosis comes to a halt at 2 hr in the duodenum and at from 4 to 6 hr in the remainder of the small intestine. It is difficult to determine when mitosis ceases in the colon, because the mitotic rate is normally low. Changes in the nuclei of the crypt cells follow essentially the same pattern at all levels of the small intestine but appear 12 to 24 hr earlier in the duodenum. Even before the cessation of mitosis the nuclei of the crypt cells become larger and quite vesicular. Large spherical globules appear among the crypt cells, possibly derived from cells which were unable to complete the mitotic cycle. These cell fragments contain an acidophilic and a basophilic component; the latter is Feulgen-positive, RN-ase resistant, and DN-ase susceptible, hence it must be DNA. In toluidine blue preparations the enlarged nuclei appear as lightly stained sacks which contain darkly stained chromatin fragments. In Feulgen preparations the entire nucleus stains a light pink but much less intensely than the normal nucleus.

After 6 hr of continuous irradiation the large vesicular nuclei of the crypt cells undergo a rapid change in which they become shriveled and highly irregular in shape. The shriveling is obviously caused by a reversal

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Figure 15. Duodenum from mouse exposed to 8 hr gamma irradiation (830 r). Outside of the irregularity of the mucosal epithelium there is no evidence of damage. Periodic-acid-Schiff stain, counterstained with hematoxylin.

Figure 16. Duodenum from mouse exposed to 120 hr gamma irradiation (12,500 r). The mucosal epithelium is completely gone, the connective tissue of the villi has collapsed and the only evidence of crypts are the slit-like lesions in the connective tissue. The vacuoles in the lower right are in the circular muscle layer. Periodic-acid-Schiff stain, counterstained with hematoxylin.

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Figure 17. Ileum from mouse exposed to 8 hr gamma irradiation (830 r). Periodic-acid-Schiff stain, counterstained with hematoxylin.

Figure 18. Ileum at 120 hr (12,500 r). Periodic-acid-Schiff stain, counterstained with hematoxylin.

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Figure 19. Colon at 8 hr (830 r). Periodic-acid-Schiff stain, counterstained with hematoxylin.

Figure 20. Colon at 120 hr (12,500 r). Periodic-acid-Schiff stain, counterstained with hematoxylin.

of the original swelling but also probably involves a loss of nuclear sap. The cytoplasm becomes extremely vacuolated with large vacuoles appearing around the nucleus. Karyolysis and karyorrhexis are going on at the same time in many crypts. The nuclei are in most cases only lightly Feulgen-positive. As a cell dies the cytoplasm appears to disintegrate quite readily while the nucleus remains as a discrete identifiable structure. In many areas the nucleus, held in position by a few coarse basophilic strands, is all that remains of the original cell.

The degeneration of the crypt cells continues until scarcely a recognizable cell remains (2-1/2 to 3 days in the duodenum, 3-1/2 to 4 in the jejunum and ileum). The original crypt cell space is filled in with submucosal connective tissue probably as a result of the spreading out of existing connective tissue.

By 24 hr changes begin to appear at the tips of the villi, where cells normally would have been pushed out at the extrusion zone by newly formed cells coming up from the crypts. With no pressure forcing the old cells out of their positions, they live considerably beyond their normal life expectancy, and an essentially intact covering is maintained over the villi. LeBlond and Stevens⁽⁵⁾ found that it took approximately 1-1/2 to 2 days for a newly formed crypt cell to move out along the villus and to be extruded. In the light of this information most of the cells which were within the extrusion zone when mitosis came to a halt were ready to be thrown out. In spite of this the cells functioned perfectly well for an additional 2-1/2 to 3 days as a protective covering.

Many of the villus cells remain in position after they are essentially dead, e.g., by 3 days the cells covering the distal half of the villi of the duodenum have a highly irregular, lightly staining, cuticular border, and their cytoplasm has lost most of its basophilic material. Eventually these cells become detached from their neighbors and drop out; however, only in the duodenum (Figure 16) are the villi completely denuded and there not until the fifth day.

The submucosal connective tissue is relatively resistant to irradiation. The amount of connective tissue appears to increase; however, it is difficult to believe that the increase is real; it is more likely that with the collapse of the crypt cells and the degeneration of the villi, the connective tissue becomes more concentrated.

The muscle cells of the muscularis mucosae begin to show the effects of irradiation at about four days: the cytoplasm becomes vacuolated and the nuclei highly distorted.

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PROGRESS REPORT: GAMMA RAY TOXICITY PROGRAM

Effects of Very High Single and Repeated Gamma-Ray Dosages on
on the Survival of LAF₁ Mice

George A. Sacher, Douglas Grahn, Katherine F. Hamilton
and Jean L. Drallmeier

Two series of tests have been carried out in the very high dose range. In September 1955, survival was studied in the range 330-2500 r/day; the mice were irradiated for 12 hr each day and were housed in the cylindrical bakelite cages that are standard for duration-of-life exposure in the gamma toxicity program. Results of this test were reported previously. (1)

In December 1955, two replicate tests were made of daily dosages in the range 1650-27,000 r/day, delivered over a 22-1/2-hr period each day. These mice also were housed in cylindrical cages. Distribution of survival times and the mean after-survivals (MAS) are given in Table 9.

In the December series, survival following single dosages was also tested. Dosages ranging from 1650 to 27,000 r were administered, protracted over a 24 hr period. Survival times are tabulated in Table 10. The 1650 r dose caused no deaths until 12 days and 17% of the animals tested survived beyond 30 days.

In Figure 21 the MAS are plotted versus daily dose for the daily-exposure series and versus single dose for the single-dose series. MAS following single dosages increases with dose in the range 3750-12,000 r. The increase, from 112 to 121 hr, is significant. MAS under continuous exposure decreases monotonically as dose rate increases, but changes very slowly in the range 2500-8000 r/day. MAS following single dosages of 3750 to 8000 r is less than the MAS observed if the same daily dosage is given throughout life.

The results obtained indicate that the length of survival in the 4-5 day period is conditioned by 2 or more interacting effects of exposure. Investigation of survival in the very high dose region is being continued. Serial sacrifices were made of mice given 2500 r daily to follow histologic and hematologic changes. These will be reported separately.

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TABLE 9

Distribution of after-survival times and mean after-survival
of LAF₁ male and female mice exposed daily to Co⁶⁰ γ-rays

Time, hr	No. of Animals							
	Daily dose - kr							
	27	18	12	8	5.5	3.75	2.5	1.65
40-44	2							
48	1							
52	0							
56	2							
60	0							
64	4							
68	5							
72	8							
76	12							
80	2							
84		1						
88		3						
92		10			2		1	
96		13	1		0	1	0	
100		9	0		1	1	0	
104			5	3	3	1	0	
108			5	1	1	3	1	
112			7	6	3	1	0	1
116			7	11	8	5	5	1
120			9	4	4	5	2	4
124			2	3	1	4	0	2
128				4	3	2	3	1
132				1	2	6	1	1
136				1	2	3	3	0
140				2	1	1	2	5
144					2	1		1
148					2	0		2
152					0	1		
156					0	0		
160					1	1		
Total	36	36	36	36	36	36	18	18
MAS	67.2	92.9	111.3	116.9	119.6	122.0	121.6	128.9

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TABLE 10

Distribution of after-survival times and mean after-survival of
LAF₁ male and female mice following single γ -ray dosages
protracted for 24 hr

Time, hr	No. of Animals						
	Dose - kr						
	27	18	12	8	5.5	3.75	2.5
40-44							
48							
52							
56							
60							
64							
68							
72							
76							
80							
84							
88							
92	1			1			
96	3			0	1	1	
100	0			0	0	2	
104	4			2	1	0	
108	1	2		0	3	0	
112	1	1	1	0	5	4	1
116	1	1	1	2	4	1	1
120	0	3	3	1	1	2	2
124	0	4	5	4	1	1	1
128	1	0	1	2	0	0	1
132		1	0		1	0	2
136			0		1	0	0
140			1			0	1
144						1	1
148							0
152							1
196							1
Total	12	12	12	12	18	12	12
MAS	103.0	117.3	121.0	115.0	112.4	112.0	132.8

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Figure 21. Effects of single and repeated exposures of 1650 to 27,000 r on the survival of LAF₁ mice. Solid line connects points for daily exposure to dosages indicated on abscissa. Dashed line connects points for survival following single dosages as indicated on abscissa. Daily dosages delivered continuously except for 15 min interruption every 4 hr. Single dose given in 24 hr, also interrupted as above.

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THE EFFECT OF IONIZING RADIATION ON FERTILITY IN THE FEMALE MOUSE

Margaret H. Sanderson and S. Phyllis Stearner

Investigations on fertility, mean litter size, and sex ratio of offspring of female LAF₁ mice following total body X-ray exposure have continued. Matings, which were begun one month after exposure of 70-day-old mice to 10, 25, 50, 75, and 100 r were made monthly until the tenth month, when the trials were bimonthly.

Data on litters produced since the beginning of this series are presented in Table 11. Data from the first nine matings, submitted in previous progress reports, (1,2) are again included for comparison. Control animals demonstrated a gradual decrease of per cent parity and average litter size with increasing age. At 14 months after exposure to 10 and 25 r, only a small number of mice were still producing litters. The incidence of observed abortions and the per cent of nulliparous animals is noted in the table.

The sterilizing dose of total-body X-ray for the LAF₁ female mouse appears to be between 50 and 75 r. Russell and Spear⁽³⁾ have reported that decreased numbers of ova are produced after the third postirradiation week. Possibly the first mating of the 75 r group was at the time when the last of these ova were available for fertilization. The reduced litter size and altered sex ratio, as seen in the mice exposed to 50 r, has been attributed to increased embryonic mortality by Russell *et al.*⁽⁴⁾ The mice in this group were sterile 5 months after exposure.

Results indicate that exposure to 25 r shortened the average reproductive period in the mouse, and litter size was decreased. Exposure to 10 r produced little early effect, but the incidence of pregnancy in the later matings decreased somewhat more rapidly than that of controls.

Finkel, *et al.*⁽⁵⁾ have reported a high concentration of Po²¹⁰ in the ovary of CF No. 1 mice for 10 days following intravenous injection of 25 μ c/kg. To ascertain the effect on fertility, litter size, and sex ratio of offspring, a series of 95 LAF₁ female mice has been injected intravenously with Po²¹⁰. These mice will be mated monthly beginning 30 days after injection.

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TABLE 11

Per cent parity, litter size, and sex ratio of offspring
of mice exposed to total-body X-ray before mating

Dose	Controls	10 r	25 r	50 r	75 r	100 r
<u>Mating 1</u>						
No. animals	71	48	32	32	32	32
% parous	68	58	53	25	16	0
% observed abortions	0	0	0	0	0	0
Average litter size	7.7	7.5	7.2	4.1	4.0	
% males	44.4	49.5	45.0	37.5	40.0	
<u>Mating 2</u>						
No. animals	71	48	32	32	32	32
% parous	58	62.5	69	22	0	0
% observed abortions	0	0	0	0	0	0
Average litter size	8.4	8.7	8.1	2.3		
% males	45.9	46.2	49.7	53.3		
<u>Mating 3</u>						
No. animals	70	48	31	32	31	32
% parous	63	54	55	25	0	0
% observed abortions	0	4	3	0	0	0
Average litter size	7.7	8.8	9.4	2.0		
% males	47.5	44.3	47.5	50.0		
<u>Mating 4</u>						
No. animals	65	46	25	29	31	32
% parous	46	41	40	6.9	0	0
% observed abortions	0	10.9	0	3.4	0	0
Average litter size	7.1	9.2	7.9	1.5		
% males	45.3	45.4	40.5	33.3		
<u>Mating 5</u>						
No. animals	63	45	25	29	31	32
% parous	59	53	40	0	0	0
% observed abortions	0	0	0	0	0	0
Average litter size	9.0	8.9	8.6			
% males	48.2	41.1	45.3			
<u>Mating 6</u>						
No. animals	63	44	25	29	30	32
% parous	50	39	36	0	0	0
% observed abortions	0	2.6	0	0	0	0
Average litter size	9.3	7.9	8.8			
% males	50.7	43.7	41.8			

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TABLE 11 (Contd.)

Dose	Controls	10 r	25 r	50 r	75 r	100 r
<u>Mating 7</u>						
No. animals	63	44	25	29	30	32
% parous	48	48	32	0	0	0
% observed abortions	0	0	0	0	0	0
Average litter size	8.2	7.5	8.3			
% males	46.2	43.9	47.0			
<u>Mating 8</u>						
No. animals	57	44	25	29	27	32
% parous	49.1	36	24	0	0	0
% observed abortions	0	0	0	0	0	0
Average litter size	8.2	7.5	8.0			
% males	47.2	46.7	43.8			
<u>Mating 9</u>						
No. animals	55	42	25	29	26	32
% parous	52.7	50	36	0	0	0
% observed abortions	0	0	0	0	0	0
Average litter size	8.1	6.4	5.2			
% males	50	40.7	36.2			
<u>Mating 10</u>						
No. animals	54	42	25	27	25	30
% parous	40.7	31	32	0	0	0
% observed abortions	0	0	0	0	0	0
Average litter size	7.1	6.4	4.8			
% males	49.7	51.8	63.2			
<u>Mating 11</u>						
No. animals	38	40	25	27		30
% parous	50	35	12	0		0
% observed abortions	0	0	0	0		0
Average litter size	5.9	5.6	5.0			
% males	44.2	50.6	60			
<u>Mating 12</u>						
No. animals	37	40	25	27		30
% parous	37.8	15	4	0		0
% observed abortions	0	0	0	0		0
Average litter size	3.5	4.7	2.0			
% males	51	53.6	50			
% Nulliparous , 12 Matings		0.0	6.3	43.8	84.4*	100

*10 Matings

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AN ANALYSIS OF THE DEPENDENCE OF THE RADIATION RESPONSE IN THE CHICK ON DOSE AND EXPOSURE TIME

S. Phyllis Stearner and Sylvanus A. Tyler

To investigate the effects of dose and exposure time on acute mortality (0-30 days) following γ irradiation, 2500 chicks (3-4 days post-hatching) were exposed to doses of Co^{60} γ rays ranging from 600 to 2400 r, given over periods of 0.5 to 8.0 hr. Approximately 36 birds were included in each dose-time group. Mortality following irradiation fell into 2 pathologically distinct periods: 0-2 days (first period) and 3-12 days (second period). After 12 days only scattered deaths occurred.

The treatment groups were divided into categories (m) on the basis of equivalent mortality in the first period, and the statistical analysis of both periods was based on these groups. The relation between the total dose delivered (D) and the corresponding exposure time in minutes (t) for dose-time pairs producing equivalent effects in the first period (for initial mortality ranging from 10 to 90%), shown in Figure 22, can be described by a linear equation

$$D = a_m + b_m t \quad (1)$$

where a_m and b_m are constants of the line for the effect (m) and can be estimated by least squares. The parameter a_m represents the dose accumulated at the end of the exposure and may be assumed to equal that dose which, if given instantaneously, would be required to produce effect (m) in the first period. The rate at which radiation-induced reactions leading to early mortality (m) are reversed is equal to b_m . The ratio of the minimal instantaneous dose (a) to the reversal rate (b) for all mortality levels (m) is constant and approximately equal to 140 min, suggesting that in order to survive the animal must be able to reverse the total accumulated dose within 140 min after completion of the exposure.

The mortality in the second period was also investigated on the basis of categories of equivalent mortality in the first period. Equation 1 shows that for each category the total dose and the time over which it was delivered are linearly dependent, so that in the second period the variable total dose can be used instead of exposure time. A plot of the log of the per cent of these living on day 3 that survived the second period against total dose also yielded a series of approximately straight lines (Figure 23). On the basis of this relation between mortality in the first period and dose effect on mortality in the second period, a model for a possible mode of action has been developed.

Figure 23. Dosage survival curves for the second period (3 - 12 days) based on groups of equivalent mortality in the first period. S_3 = 3-day survivors.

Figure 22. Relation between total dose and exposure time for equivalent mortality in the first period (0 - 2 days). The 47.6% line is estimated from the relationship existing between constants of the set of lines.

We postulate that irradiation produces an effect "A" (no implications are made at this time as to the nature of this effect) at a rate proportional to intensity of exposure and that effect "A" is reversed or inactivated at a rate dependent not on any quantitative measure of "A" but on the ability of the animal. This rate is represented by b in Equation 1, and the range of b for 80% of the population (10 - 90% early mortality) is about 4.5-7.5 r/min. The total effect "A" that is not reversed reaches a maximum at the end of the exposure. This maximum "A" exerts an irreversible effect on a sensitive system that leads to early death in the chicken and that also influences mortality in the second period. Irradiation produces another effect "B," the amount of which is proportional to total dose delivered. This effect is irreversible; it produces lesions leading to mortality in the second period but does not influence mortality in the first period. Following a dose of radiation, mortality in the second period is determined both by the maximum amount of effect "A," which is rate-time dependent, and by the total amount of effect "B." If mortality in the first period is considered an indicator of degree of insult to the animal, an insult that is proportional to the effect of maximum "A," one would expect the rate of mortality in the second period to be proportional to the initial insult. This relationship is shown in Figure 23. Thus, survival in the second period is determined by the interaction between degree of initial insult (amount of effect "A"), and effect "B," the amount of which is proportional to the total dose delivered.

It is recognized that there are alternative interpretations of the relation between dose and exposure time in the radiation response of the chick. These may yield other biological models and are being considered. To test specific inferences from this model and to provide more data for an investigation of other models, several additional experiments have been planned.

PROGRESS REPORT: NEUTRON TOXICITY PROGRAM

The Effect of Single Irradiation with Fission Neutrons
on Fertility in the Female Mouse

Howard H. Vogel, Jr., John W. Clark, and Donn L. Jordan

As a control for the effects of irradiation on fertility, a study was made of the number of young born in the first litters of unirradiated CF No. 1 female mice. In Figure 24 a graph is presented on the distribution of litter size in 124 first litters of such mice, 2 to 6 months of age. The average among these unirradiated mice was almost 10 young per litter.

Preliminary Experiments (at the CP-3 Reactor). After whole-body irradiation with single doses of fission neutrons within the acute lethal range (181-245 rep), 20 female CF No. 1 mice were tested for fertility by placing two irradiated and two control (unirradiated) females in a cage with a fertile male mouse for a period of four days. Tests for fertility were made at 2-1/2, 4, and 5-1/2 months after irradiation, and no data were counted unless the male proved to be fertile. None of the neutron-irradiated females produced any young.

In a second experiment, 50 female mice were irradiated with a lower total dose (101-137 rep of fission neutrons) and were tested for fertility at 2 weeks after exposure. Twenty litters were born. However, the mean litter size was only 3.8 in comparison with 9.4 in the unirradiated control mice. It was thus obvious, that, although permanent sterility was not yet evident, the size of each litter was markedly reduced following a neutron exposure in this range.

A third series of mice was then exposed to a dose of fast neutrons (33-59 rep) and tested for fertility at 7 and at 9 weeks after irradiation. None of the 20 irradiated mice gave birth to any young, although 10 controls produced litters.

Further Studies (at the CP-5 Reactor). Mice were exposed at CP-5 to single acute doses of 46 and 93 rep. Figure 25 compares the number of young born per litter in neutron-irradiated females and in unirradiated controls. Cumulative results are presented from four mating tests in which males were introduced to cages containing 2 irradiated and 2 control females at 3, 10, 17, and 24 days after the neutron exposure. A dose of 46 rep of fission neutrons does not decrease the per cent parity, but does decrease the average litter size, at least during the three-week period after the exposure. The higher dose of 93 rep decreases both the number of pregnancies and the mean litter size. Data from one other neutron exposure (32 rep, delivered in 20 min) are listed in Table 12. After the first month following neutron irradiations, only two of the female mice produced any young.

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Figure 24

Figure 25

TABLE 12

Effects of 32 rep of fission neutrons on fertility of CF No. 1 female mice

Mating		Number parous/number tested		Mean litter size	
Fertility test	Days after irradiation	Irradiated	Control	Irradiated	Control
1	4	7/10	8/10	8.9	9.9
2	11	7/8	6/8	7.7	9.0
3	18	6/10	7/10	7.5	9.3
4	25	4/8	6/8	9.5	7.8
5	32	1/4	2/4	6.0	7.0
6	39	0/6	4/6	0	9.8
7	84	1/8	7/8	2.0	9.1

In another experiment, 50 female mice were exposed for 5 min to a single acute dose of fission neutrons in the biological radiation facility at CP-5 (estimated dose 11.5 rep). They were tested for fertility 1 day after exposure and at weekly intervals thereafter for three successive weeks. During this period 34 of the irradiated mice produced litters with a mean of 8.1 young per litter in comparison with a mean of 9.5 in 24 control litters, sired by the same males. However, none of the control litters contained less than 7, while 23% of the litters from irradiated females were less than 7 in number. Thirty-four of the 40 (85%) neutron-irradiated mice that were tested produced young, whereas only 71% of the unirradiated mice gave birth in this period. Thus, it is clear that this dose of neutrons did not lower the per cent of pregnancies during the 3-week period following the exposure.

Although this low neutron dose showed little immediate effect on fertility (except for the slightly lower number of young per litter), these mice were completely sterile when retested at 5 months after exposure: no young were produced by 10 irradiated females that were tested, whereas 7/10 of the controls gave birth to normal litters.

According to Sanderson and Stearner, (1) female mice (LAF₁), after 50 r of whole-body X-radiation, produced no offspring after a 4-month period. Thus, the RBE (n/X) for permanent sterility in female mice after single acute irradiation would appear to be at least 4 or 5.

Further investigations are underway using lower doses of fast neutrons.

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PROTECTION OF NUCLEOPROTEIN FROM X-RAY-INDUCED VISCOSITY REDUCTION

Robert N. Feinstein

The following two facts were reported earlier: (a) The addition of cysteine to alkaline solutions of nucleoprotein (thymus chromosomes) caused a considerable increase in viscosity, and (b) the presence of cysteine in X-irradiated alkaline nucleoprotein solutions largely prevented the reduction in viscosity brought about by the irradiation in a corresponding solution not containing cysteine.⁽¹⁾ The hypothesis was tentatively proposed that a similar "stabilization" of nucleoprotein in vivo might be at least partly responsible for the protection afforded by cysteine against X-ray lethality.⁽²⁾

Dale⁽³⁾ recently commented on these experiments and suggested that the above hypothesis would be reinforced if it could be demonstrated that 1 M NaCl, which also increases the viscosity of nucleoprotein, similarly protects nucleoprotein from the viscosity reduction normally induced by X-irradiation. This experiment has been completed, and it has been found that 1 M NaCl does not so protect nucleoprotein solutions. The suggestion that nucleoprotein "stabilization" is of importance in cysteine protection in vivo is thus weakened.

Experimental

Thymus chromosomes were prepared by the method of Mirsky and Ris,⁽⁴⁾ except that 1 ml of the final suspension of chromosomes stored in 0.14 M NaCl represented 0.5 g of original glandular material. Immediately before use, the stock suspension was diluted with 0.14 M NaCl in such a fashion as to make the viscosities in NaOH or 1 M NaCl approximately equal. Thus, for the alkaline solutions, 10 ml of the stock suspension was mixed with 4 ml 0.14 M NaCl, and this mixture was mixed with an equal volume of 0.1 M NaOH; 5 ml of this solution was then mixed with 1 ml of water or 0.06 M cysteine. When the chromosomes were to be dissolved in 1 M NaCl, one volume of the stock suspension was mixed with an equal volume of 0.14 M NaCl, and one volume of this mixture was mixed with an equal volume of 2.0 M NaCl. Cysteine was freshly prepared and brought to pH 8.0 immediately before irradiation.

X-irradiation was performed with a GE Maximar 250. Radiation factors used were: 250 kvp, 15 ma, no added filter, HVL 0.5 mm of copper, target distance 7.5 in. The rate of irradiation was 1206 r/min for a total dose of 50,000 r, and 6 ml aliquots of solutions were irradiated at room temperature in 10 ml beakers covered with parafilm.

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Viscometry was done at 37°C using standard Ostwald viscometers with a flow time (5.0 ml of distilled water) of about 30 sec. Aliquots of nucleoprotein solutions were placed in the viscometers within 2-3 min after completion of the irradiation. Then 10 min was allowed for the solutions to reach the temperature of the bath before the first viscosity determinations were begun.

The results are given in Table 13. Because of the usual increase in viscosity with time, values are presented both at zero time and after four hours.

TABLE 13

Protective effect of cysteine or of 1 M NaCl on viscosity of X-irradiated nucleoprotein

	1 <u>M</u> NaCl		NaOH; no cysteine		NaOH; cysteine	
	Control	X-rayed	Control	X-rayed	Control	X-rayed
Zero time						
Intrinsic viscosity η_i :*	15.5	12.2	11.1	7.9	27.2	25.9
% reduction in η_i :		21		29		5
Four hours						
Intrinsic viscosity, η_i :	22.4	13.5	15.7	8.4	26.6	24.9
% reduction in η_i :		39		46		6

*Intrinsic viscosity, η_i = relative viscosity - 1.

Discussion

The conclusion seems clear from the experiment described, that even though 1 M NaCl greatly increases the viscosity of nucleoprotein solution, it does not protect such solutions from the viscosity-reducing effects of X-rays as does cysteine. Although this is presumptive evidence that the mechanism of cysteine protection against radiation lethality is not some form of "stabilization" of the nucleoprotein in vivo, it cannot be considered proof. The possibility remains that the stabilization of the nucleoprotein by 1 M NaCl in some way differs from that effected by cysteine.

70References

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PROGRESS REPORT: MECHANISMS OF LEUCOCYTE REGULATION

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and Robert L. Straube

We reported previously⁽¹⁾ that blood neutrophil recovery in the dog appears to be qualitatively similar whether leucopenia is achieved by exchange transfusion with leucocyte-depleted blood or by injection of leucocyte antiserum to remove the cells *in vivo*. Recovery in these instances is accomplished mainly by the release of nonsegmented cells from the bone marrow. Analysis of the distribution of presumed transition states of mature neutrophils (nuclear lobe count) is perhaps consistent with this view, since there is no indication of a bimodality during recovery. The mean nuclear lobe counts are presented in Table 14.

A relationship between neutropenia induced by antiserum and the appearance of nonsegmented cells during recovery is shown in Figure 26. This consideration suggests that the extent of release of nonsegmented cells, mainly band forms, is related more to the relative (per cent change) than to the absolute decrease in neutrophils. This is consistent with the view that the number of mature cells available for release from marrow is a function of the normal steady state level in blood.

The mechanisms of compensation for the decline in cells may involve a suppressor feedback between the mature neutrophils and blood-forming sites. This can take several forms, e.g., chemical or physical, and may involve distinct mechanisms for cell release and new cell formation. This proposition is being explored in experiments in which blood from a leucopenic X-irradiated dog is shunted through the isolated extremities of a normal dog. In these studies, blood circulates through polyethylene tubing from the aorta or femoral arteries of the irradiated dog to the distal part of the aorta of the normal animal. The blood is returned through similar tubing connecting the vena cava of the latter and the vena cava or femoral veins of the former. Provision is made for sampling of blood to and from the perfused part and for measurement of blood flow. Complete blood counts and hematocrit readings were made at frequent intervals with occasional measurements of blood volume; the marrow was examined upon termination of the experiment. The irradiated dog received a total-body exposure of 400 r, 5 to 6 days prior to cross-circulation. Leucocyte antiserum was given during this interval to hasten the exhaustion of marrow reserves.

In the initial experiments, the perfused area (pelvis and hind legs) was not isolated surgically; however, this was done in the more recent preparations because of the indeterminate contribution by collateral circulation. Despite application of various tourniquets, binding of the isolated

TABLE 14

Neutrophil lobe count (weighted mean)

Antiserum group	Before treatment	Time, hours								
		0.125	0.5	1	2	3	4	6	24	48
1:1	3.42-3.37	2.07	2.17	2.00	1.59	1.39	1.55	1.99	-	-
1:1	4.08	2.06	2.21	2.02	1.91	1.98	1.73	1.59	2.84	3.00
1:10	3.66	3.03	2.61	2.36	2.06	1.79	1.51	1.99	2.92	-
1:10	3.30-2.94	3.12	2.00	1.85	1.57	1.48	1.43	1.98	1.95	2.58
1:100	3.27-3.10	3.06	2.53	2.65	2.39	2.28	2.23	2.19	3.13	3.03

part with elastic bandage and intermittent electrical stimulation of nerves and muscles, there is a small but continuous blood loss both from the amputation site and into the perfused part. From the point of view of the circulating blood volume of the intact dog, this can be compensated for, to some extent, by infusion of fluids, e.g., dextran.

Although there are technical difficulties in maintaining the preparation for more than a few hours, the first hour or two of perfusion is relatively uncomplicated. The results of a few experiments give some indication of the number of mature and immature neutrophils available for prompt release to the circulation. The sequence of changes in an intact and isolated preparation is presented in Figures 27 and 28. The venous-arterial difference, which represents the contribution from the perfused part, is shown in Figure 29. There is a rapid increase in the number of nonsegmented cells (band and metamyelocyte) that are released. These constitute about 85% of the total neutrophils after 30 min of perfusion (Figure 30).

The large volume of neutrophils released during the first hour is shown in Table 15. The calculation is based on the mean difference between venous (donor from the standpoint of the origin of the cells) and arterial (recipient) blood and mean blood flow. These findings receive qualitative support from comparison of changes in the myeloid-erythroid ratios of perfused and unperfused marrows. Most of the cells contributed by the perfused part are apparently lost rapidly from the circulation. The various traumata incidental to the procedure doubtless contribute to this. There is some indication of a preferential loss of segmented neutrophils. These data reveal the vast number of cells that are available in marrow, particularly when we reflect that the perfused area represents only about 25 to 30% of the body weight. The order of cell numbers is consistent with theoretical considerations.^(2,3)

Figure 26. Relationship between neutropenia and release of nonsegmented cells. (Neutropenia is expressed in terms of absolute decrease or per cent decrease from the control blood level. Each point represents the mean of determinations on 3-5 dogs; ranges are signified by dashed lines.)

Figure 27. Cross circulation of pelvis and hind legs (intact preparation) with leucopenic blood.

Figure 28. Cross circulation of isolated pelvis and hind legs with leucopenic blood.

Figure 29. Neutrophils released from pelvis and hind legs during first hour of perfusion.

Figure 30. Relative change in nonsegmented neutrophils during perfusion. (Each symbol represents a single animal.)

TABLE 15

Neutrophil release during perfusion of pelvis and hind legs

Experiment	Donor-recipient (Av/mm ³)		Neutrophils x 10 ⁻⁹ released during first hour*		<u>Cells released</u> <u>Basal blood level</u>
	Segmented	Nonsegmented	Segmented	Nonsegmented	
456-62 Isolated	738	1171	13.3	21.1	4.9
464-66 Isolated	193	615	3.5	11.1	2.1
452-58 Intact	991	2645	17.8	47.6	9.3

* Mean blood flow = 300 ml/min.

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EFFECT OF NONIRRADIATED, CENTRIFUGED PROTOPLASM
ON SUPRALETHALLY X-IRRADIATED GIANT AMOEBAE
PART III

Edward W. Daniels

The giant multinucleated amoeba, Pelomyxa illinoisensis, dies within 3 to 8 days following lethal X-ray exposure (15 kr). If nonirradiated protoplasm from another animal is injected by micromethods into the exposed cell, death is prevented.^(1,2) Survivors give rise to mass cultures. The present work is concerned with the effect of adding centrifugal and centripetal halves of non-irradiated cells to X-irradiated (24 kr) amoebae.

Nonirradiated amoebae were centrifuged at forces which ranged from 700 to 30,000 g. The stratified cell was halved, and each portion was injected into a different X-irradiated cell. The heavy, nucleated, centrifugal portions brought about recovery regardless of the gravitational force used. The light, centripetal portions obtained from cells subjected to forces less than 6,000 g also brought about recovery. However, the per cent of injected cells which survived was reduced in a linear manner as the centrifugal force was increased from 1 to 6,000 g (Fig. 31).

Figure 31. Effect on survival of injecting centripetal (light) portions of non-irradiated Pelomyxa illinoisensis into supralethally X-irradiated cells of the same species.

Cytological studies of X-irradiated cells show that nuclei and mitochondria persist until cell death without obvious morphological changes, although there is some nuclear swelling. Considerable evidence has been obtained which indicates that the irradiated nuclei completely recover from radiation damage in treated cells. X-irradiated cells which received nonirradiated protoplasm were fixed at various times during the first post-radiation mitosis period. All of the nuclei in these experimental cells were synchronous, i.e., in the same stage of mitosis, which is also the normal finding in nonirradiated cells. Further evidence of the participation of irradiated nuclei was obtained following the use of centripetal portions of nonirradiated cells which contained few if any nuclei. Cell division of irradiated cells occurred relatively soon following treatment with these light halves, provided the force did not exceed some 5,000 g. However, cell division rarely occurred in the isolated light halves themselves, and then only after a long lag period. Nuclear division in the treated cells was synchronous, and normal in appearance, and it occurred relatively soon after treatment.

At the present time it appears that some component which migrates toward the centrifugal pole is necessary for recovery. The pattern of migration is quite similar to that of the mitochondria. However, the need on the part of the irradiated cell for these nonirradiated mitochondria is somewhat questionable since no difference in the morphology of the mitochondria in irradiated and nonirradiated cells has been observed. It is possible that their function is impaired. Bipyrarnidal and plate-like crystals migrate close to the nuclei and it does not appear that these are a necessary part of the treatment. More dense constituents of the nonirradiated protoplasm such as heavy spherical granules and food vacuoles are not essential.

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APPLICATION OF GAMMA-RAY SPECTROMETRY TO MULTIPLE-TRACER STUDIES

Philip F. Gustafson and William M. Westfall

In some phases of biological or medical research as well as in radiochemical analysis and related work it may be desirable to make quantitative determinations of two or more isotopes simultaneously. If the radioisotopes involved are γ emitters, such measurements may be carried out using a NaI(Tl) well counter in conjunction with a single-channel, pulse-height analyzer. Such a combination has been tried successfully by Francis and Bell⁽¹⁾ and independently by Hine.⁽²⁾ The discrete γ -ray spectra of many isotopes provide keys to their identity, since by counting in the region of the photoelectric peak it is possible to differentiate between various γ -rays on the basis of their energies.

The instrument used in our work was a $2 \times 2\text{-}1/4$ in. NaI(Tl) crystal with a $3/4 \times 1\text{-}1/8$ in. well, connected to a conventional linear amplifier and single channel analyzer. Figure 32 shows the γ -ray spectrum of Mn^{54} which emits a single 0.84 Mev γ -ray. In order to integrate the counts occurring in the photo-peak the analyzer base line was set in the valley between the Compton distribution and the photo-peak proper (about 75 on the base line setting). The window width is then progressively increased until the counting rate reaches a plateau, as indicated in the insert. This "optimum window width" is the minimum width within which the photo-peak of any energy. Determining the window in this manner greatly minimizes errors due to slight changes in the photo-tube voltage.

If both Mn^{54} and Cr^{51} (.32 Mev) must be measured, the optimum window is found for each individual element. Although all Mn^{54} γ -rays possess 0.84 Mev energy, they do not all interact by photoelectric absorption within the crystal. Many will undergo Compton absorption resulting in pulses of energy lower than those in the photo-peak, and some of these pulses will occur within the Cr^{51} window. For this reason Mn^{54} is counted in the Cr^{51} window as well as within its own optimum window, and the fraction of Mn^{54} counts appearing in the Cr^{51} window relative to those in its own must be established. In this particular case the fraction is 0.57. Then, when a mixture of Cr^{51} and Mn^{54} is counted, the counts in the Cr^{51} window which we will call A are given by

$$A = \text{Cr} + .57 \text{ Mn}$$

while those in the Mn^{54} window, B, are

$$B = \text{Mn}$$

Figure 32. Gamma-ray spectrum of Mn^{54}

Figure 33. Individual spectra of Cr^{51} , Mn^{54} , and Zn^{65} as well as the composite

Figure 34. Spectra and windows used for a mixture of Cr^{51} and I^{131}

Figure 35. Spectra of Zn^{65} and Co^{60} and the windows used

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From the count in the B window it is possible to determine how many of the counts in the A window are due to Mn, and thus the actual number of Cr counts is obtained.

Figure 33 shows the individual spectra of Cr^{51} , Mn^{54} , and Zn^{65} as well as the sum as it would appear in the actual mixtures; the optimum windows are indicated by A, B, and C respectively. When analyzing a mixture the counts in A, B, and C have the following composition:

$$(1) A = \text{Cr} + .57 \text{ Mn} + .568 \text{ Zn}$$

$$(2) B = \text{Mn} + .472 \text{ Zn}$$

$$(3) C = .023 \text{ Mn} + \text{Zn}$$

Solving (2) and (3) for Mn and Zn it is then possible to subtract the Mn and Zn contribution to A and thus to arrive at the count due to Cr alone. The accuracy attained in the triple tracer study was within 3% for each isotope. To obtain activities in terms of microcuries it is necessary only to place a calibrated source in the well and to count in the optimum window for that isotope; this gives a figure of counts/min/ μc which may then be compared with the unknown activities. The volumes must be the same in the standard and unknown mixtures; a useful procedure is to dilute to a standard volume (of the order of a few cc).

The Cr^{51} - Mn^{54} - Zn^{65} mixture constitutes a rather idealized situation in which the single γ -rays emitted by each isotope are rather widely spaced in energy. The method was tested more rigorously by using two other mixtures, each composed of two isotopes for which the principal photo-peaks overlapped within the resolving power of the system. Figure 34 shows the spectra and windows used for a mixture of Cr^{51} and I^{131} . The A and B windows were similar to those used by Francis and Bell,⁽¹⁾ whereas A and A' represented a slight variation, in which only the window width was changed while the same analyzer base line was retained. In both methods the accuracy was 2%. Figure 35 depicts the spectra of Zn^{65} and Co^{60} and the windows used; here again accuracies of the order of 2% were obtained.

Mixtures such as these actually constitute a simulated radioactive series in which the various members emit γ -rays of different energies and intensities. Therefore such series also lend themselves to such analysis with equal facility.⁽³⁾ There would seem to be no real limit to the number of isotopes which can be analyzed simultaneously in this manner if the investigator has the time and patience. Admittedly a multi-channel analyzer would reduce materially the time required for taking data, but the analysis would remain essentially the same.

A subject for further investigation would be the effect of variation in solids present in the solution upon the production of bremsstrahlung, if

β -rays are emitted also. The effect should not be serious, in the light of work done on P^{32} by Loevinger⁽⁴⁾ as well as preliminary work done here, in which the efficiency for bremsstrahlung production was found to be very small (of the order of 1%). It is conceivable that such effects might reach the order of a few per cent if the number of γ -rays per β -ray were very small. The practical limitations of the method in the presence of widely different activities remain to be established.

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THE EFFECT OF HEAT AND RADIATION UPON THE CONDENSATION OF CARRIER-FREE, RADIOACTIVE PHOSPHORIC ACID ($\text{H}_3\text{P}^{32}\text{O}_4$)

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Some of our current observations indicate that special care must be exercised in the preparation, use, and analysis of radioactive phosphate tracer in order to prevent the formation of condensed phosphates. These observations pertain particularly to the neutron activation, to the preparation of concentrated solutions of tracers, to the evaporation of the solutions, and to the methods of analysis.

In the course of our investigations of radioactive phosphate ($\text{H}_3\text{P}^{32}\text{O}_4$) by electrochromatography, radioactive contaminants or alteration products were frequently encountered. Some of these products were observed in radioactive phosphate prepared at Oak Ridge National Laboratory.⁽¹⁾ Several additional radioactive substances were found in various crystalline phosphate compounds that had been exposed to neutrons in the Argonne reactor.⁽²⁾

As indicated by our early electrochromatographic analyses, the Oak Ridge phosphate, prepared carrier-free from elemental sulfur by an (n,p) reaction followed by oxidation, often contained two substances that migrated much slower than orthophosphate. One of these substances, first isolated in 1952, was converted into orthophosphate by hydrolysis with mineral acids.⁽¹⁾ The other, which was present in small quantities, remained at the starting point in the electrical migrations.

Recent analyses of highly active H_3PO_4 obtained from Oak Ridge have again revealed two substances in addition to orthophosphate. But now one of these migrated faster than orthophosphate in paper moistened with 0.1 M lactic acid, and it proved to be pyrophosphate. The other, like one of those detected four years ago, scarcely migrated from the point at which it was added to the moist paper. This lack of migration was due, in part at least, to the strong sorption by the paper, because the activity was not eluted from the paper by extraction with water, lactic acid (0.1 M), or hydrochloric acid (1 M).

The crystalline phosphate preparations that were irradiated with neutrons, dissolved, and examined electrochromatographically contained, primarily, radioactive substances that migrated faster than orthophosphate. These several radioactive products were of two kinds. Some were hydrolysed to orthophosphate by heating with hydrochloric acid and were presumably condensed phosphates. Others were converted into orthophosphate by

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oxidation, as with nitric acid and hydrogen peroxide, and two of these were identified as phosphorous and hypophosphorous acids by comparative migrations.

For some time it was believed that all the radioactive contaminants of the neutron-irradiated orthophosphates were formed by instantaneous, secondary, chemical reactions induced by the primary nuclear reactions.^(1,3) As experience has accumulated, however, there have been indications that some of these hydrolysable products may be formed by irradiation with the intense gamma rays and by chemical reactions independent of the nuclear reactions. Analogous chemically produced condensation reactions might also explain the formation of the slowly-migrating, hydrolysable contaminants in the carrier-free orthophosphate prepared from sulfur.

It has been known for a long time that heating of phosphoric acid or the acid phosphates will produce a series of condensed phosphates. Of these condensed phosphates, pyrophosphate, trimetaphosphate, and tetrametaphosphate are the simplest. Our investigations have shown that these simple condensed phosphates migrate faster than the orthophosphate itself in the electrochromatographic separations. Some of the polycondensation products, by contrast, migrate slower than the orthophosphoric acid.⁽²⁾

Recently there have been reports indicating that relatively mild conditions may promote the formation of condensed phosphates from phosphoric acid and from solutions of phosphate salts. For example, when labeled phosphoric acid was heated in a platinum vessel at 100°C for 24 hr or more, and dried in vacuum at 2 to 3 mm Hg for 16 hr, some 4% was converted into pyrophosphate. Heating at 176°C produced pyrophosphoric acid (40%), triphosphate (10%), tetrphosphate (2%), and pentaphosphate (0.2%). Analyses were performed with anion-exchange columns, in which the more condensed products migrated slower than the less condensed products (0.05 to 0.5 M KCl as wash liquid).⁽⁴⁾

The formation of complex condensed phosphates under mild conditions was also observed when magnesium tetrametaphosphate was formed from the sodium salt in water and precipitated with methanol plus acetone. Some of these condensation products were also found in 0.1 M solutions of phosphate salts that were allowed to stand for some 165 days, that were heated with acids, or that were heated with alkalis. Detection of the condensed products was based upon the use of paper chromatography and electrochromatography.⁽⁵⁾

As a rule, the mobility of the condensed polyphosphates in paper chromatography and in columns of ion exchangers decreased with the degree of condensation,^(3, 4, 5, 6, 7) whereas the electrical mobility increased with condensation, except for the very highly condensed polyphosphates^(3, 5) whose migration would be hindered by their very strong sorption on the paper.⁽⁶⁾

To confirm our earlier observations, to find sound explanations for the complexity of tracer phosphate, and to avoid spurious alteration of phosphate in solution, we have now studied some of the changes produced by treatment of purified, carrier-free phosphate under various conditions. In this investigation, there have been three principal stages; namely, purification and recovery of the carrier-free phosphate, exposure of the phosphate to various conditions, and analysis of the mixtures produced by the different conditions.

Preparation of carrier-free phosphate. Experiments with the neutron irradiation of phosphate compounds confirmed that a mixture of radioactive products was usually produced. This mixture was difficult to separate completely by electrochromatography in paper moistened with dilute lactic acid. It could not be converted into orthophosphate without extensive hydrolysis with acid and oxidation with nitric acid and peroxides, and the radioactive phosphate always contained much inactive carrier.

To obtain pure phosphate free of the carrier and the condensation products, we purified the carrier-free tracer obtained from Oak Ridge National Laboratory. For this purpose, the highly active tracer (about 30,000 μc , or 30 mc) was submitted to electrical migration in a paper pad moistened with 0.1 M lactic acid.⁽¹⁾ As shown by the autograph in Figure 36, this preparation contained three principal radioactive components; namely, one in a zone which did not migrate, one which migrated rather rapidly and which proved to be identical with orthophosphate, and a third which migrated fastest and which was identical with pyrophosphate. These comparative migrations are shown in Figure 37. The material in the zone which did not migrate could not be eluted with water, 0.1 M lactic acid or 1 M HCl. It was not examined further. The principal phosphate zone was removed, eluted with water, and the elutriate was evaporated to dryness at reduced pressure. This left a yellow residue that was not decolorized by solution in nitric acid followed by evaporation at reduced pressure. The highly active residue was, therefore, made up to pH 10 with sodium hydroxide, dissolved in water, and sorbed in an anion-exchange column prepared in the chloride form.⁽⁴⁾ The sorbed radioactive phosphate was then washed with water and eluted with 0.05 M HCl until the phosphate was removed. The elutriate was evaporated and dried at room temperature under reduced pressure by means of an oil pump. The colorless residue was dissolved in 20 ml of water, yielding a solution of pH 7.0. A portion of this solution analyzed by electrical migration in paper moistened with 0.1 M lactic acid yielded a single zone of radioactive orthophosphate. This showed that all condensed phosphates and all coloring matter had been removed.

In another series of experiments, the preliminary purification by electrical migration was omitted. The neutralized solution of the tracer was sorbed directly in the anion-exchange column. As in the experiment

OAK RIDGE CARRIER-FREE RADIOACTIVE PHOSPHATE.

Figure 36. Radioautograph illustrating electrochromatographic separation of carrier-free radioactive phosphate, obtained from Oak Ridge National Laboratory. Unidentified product "A" remained at starting point. "B" was subsequently identified as $\text{H}_3\text{P}^{32}\text{O}_4$ and "C" as $\text{H}_4\text{P}_2^{32}\text{O}_7$ as shown in Figure 37.

IDENTIFICATION OF COMPONENTS OF OAK RIDGE RADIOACTIVE PHOSPHATE.

Figure 37. Radioautograph illustrating the comparative electrochromatographic identification of $\text{H}_3\text{P}^{32}\text{O}_4$ and $\text{H}_4\text{P}_2^{32}\text{O}_7$, separated from radioactive phosphate as shown in Figure 36. Starting point indicated by +. An extract of zone A from Figure 36 did not provide detectable quantities of radioactivity at A and A. B, B and C, C contained radioactive material extracted from the respective zones of Figure 36. Before migration, samples B and B were mixed with 0.01 M carrier NaH_2PO_4 ; C and C were mixed with 0.01 M carrier $\text{Na}_2\text{H}_2\text{P}_2\text{O}_7$; and Oak Ridge phosphoric acid was mixed with 0.01 M NaH_2PO_4 and $\text{Na}_2\text{H}_2\text{P}_2\text{O}_7$. In the Figure, the radioactivity is indicated by the dark zones. The location of the carrier H_3PO_4 and $\text{H}_4\text{P}_2\text{O}_7$, determined with molybdate reagent, is indicated by the lines surrounding the dark zones.

just described, a colorless tracer, free of condensation products, was obtained. In all these experiments, there was a poor recovery of the radioactive phosphate from the anion-exchange column, even after preliminary purification of the phosphate by electrical migration⁽¹⁾ or by anion exchange.⁽⁴⁾

Exposure of phosphate to various conditions. As phosphate solutions are frequently handled in glass vessels, all the experiments with the tracer were carried out in Pyrex utensils. These experiments were designed to investigate the action of heat on phosphate and on the hydrolysis of the condensed phosphates.

Because solutions of labeled phosphoric acid are frequently evaporated to dryness on a steam bath, a series of these evaporations was carried out, both with and without the addition of mineral acids. The tracer solutions (1 ml containing 0.5 mc of activity) alone or with various reagents, were placed in 30 ml beakers, which were then set in rings on a steam bath so that the lower half of each beaker extended into the steam which had a temperature of about 98°C. One sample was evaporated to dryness (about 10 min), after which it was dissolved in about 1 ml of water, and thereafter water was added at short intervals to keep the volume constant for about 30 min. Upon electrical migration, this sample yielded orthophosphate plus a large proportion of pyrophosphate. Only faint traces of other radioactive species were present. Another sample was likewise evaporated to dryness but was taken up in 0.1 M HCl and then heated for 30 min. It yielded primarily orthophosphate plus only a trace of pyrophosphate. A third sample was also evaporated to dryness but was taken up in fuming HNO₃ (1 ml) plus 30% H₂O₂ (0.25 ml). This acid solution was then evaporated to dryness, and the residue was taken up in distilled water. After 30 min on the steam bath, the solution yielded primarily orthophosphate with some pyrophosphate and traces of two substances which migrated between the orthophosphate and the pyrophosphate. A fourth sample was evaporated and treated with HNO₃ and peroxide as just described, after which it was dissolved in 0.1 N HCl and heated for 1 hr. Almost all of the activity of this sample represented orthophosphate, with only traces of the zones described in the preceding experiment.

Because solutions of phosphoric acid are often evaporated to dryness on hot plates and because the temperature of the hot plates is often not controlled, several samples were concentrated on an asbestos-covered electric plate and the products, treated in various ways, were examined electrochromatographically. To obtain some estimate of the temperature of the hot plate, a thermometer was placed in a 30 ml beaker that was then filled with sand and allowed to stand on the hot plate. The temperature remained constant at 270°C. As in the series of experiments just described, a 1 ml portion of the tracer (0.5 mc) was placed in a 30 ml beaker on the hot plate. The sample became dry in about 10 min but was allowed to remain for another 10 min, a total of 20 min; then the residue was taken up in distilled

water and heated for 30 min. Electrical migration in paper moistened with 0.1 M lactic acid revealed orthophosphate with much pyrophosphate and traces of trimetaphosphate and tetrametaphosphate. There was no activity at the starting point, and there was very little trailing of the migration zones as shown in Figure 38. A second sample evaporated and heated in the same manner was dissolved in 0.1 N HCl and boiled for 30 min. This sample provided orthophosphate with smaller amounts of pyrophosphate and little or no trimetaphosphate and tetrametaphosphate. A third sample was treated with fuming HNO_3 (1 ml) plus H_2O_2 (0.25 ml), evaporated to dryness, dissolved in distilled water, and boiled for 30 min. It provided orthophosphate with large amounts of pyrophosphate. A fourth sample treated like the one just described but finally dissolved in 0.1 N HCl provided orthophosphate plus small amounts of pyrophosphate.

Figure 38. Radioautograph illustrating the electrochromatographic examination of $\text{H}_3\text{P}^{32}\text{O}_4$ purified by ion exchange and heated in a beaker on a hot plate at 270° for 0.5 hr. The dark areas represent the locations of the radioactive products. The circles represent the comparative positions of authentic acids of phosphorus added as 0.01 M solutions of their salts and located with molybdate reagent after the migration.

Because of the uneven distribution of heat in an open beaker placed on a water bath or on an electric plate, several samples (1 ml, 0.5 mc) of the carrier-free phosphate were heated at different temperatures in an electrically heated muffle. All these samples were heated for the same time (30 min), and all were dissolved in distilled water. As shown by electrochromatography with 0.1 M lactic acid, one sample heated at 150°C yielded orthophosphate, some pyrophosphate, and much activity remaining at the starting point. Another sample heated at 200° yielded orthophosphate, much pyrophosphate and tripolyphosphate, and much activity remaining at

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the starting point. A third sample heated at 250° yielded products like those obtained from the sample heated to 200° . And a fourth sample heated at 300° yielded orthophosphate, pyrophosphate, the nonmigrating material, but little or no tripolyphosphate. In the samples heated at 200, 250 and 300° , there was much trailing of the zones, and there was indication of a minor zone a short distance ahead of the orthophosphate zone as illustrated in Figure 39. The material that did not migrate could not be eluted from the paper with water or with dilute acids.

Figure 39. Radioautograph illustrating the electrochromatographic examination of $\text{H}_3\text{P}^{32}\text{O}_4$ purified by ion exchange and heated in a beaker in a muffle at 300° for 0.5 hr. The dark areas represent the locations of the radioactive products. The circles represent the comparative positions of authentic acids of phosphorus added as 0.01 M solutions of their salts and located with molybdate reagent after the migration.

Because phosphoric acid condenses so readily, one might suspect that this condensation plus condensation with sulfuric acid might occur when solutions of sulfuric acid containing tracer phosphoric acid are evaporated. To test this possibility, 1 ml solutions of the purified phosphoric acid tracer were added to 0.5 ml of 0.5 N sulfuric acid, and the mixtures were heated for 0.5 hr in the muffle at temperatures ranging from 50 to 300° . These products, examined by electrochromatography, contained almost solely radioactive orthophosphate. Under these conditions, therefore, the sulfuric acid retards the condensation of the phosphate.

As some of the radioactive products formed by the action of neutrons on phosphates might have arisen from the action of γ -rays, purified, "carrier-free" preparations of $\text{H}_3\text{P}^{32}\text{O}_4$ were irradiated separately with neutrons and with γ -rays under comparable conditions. Pairs of samples were prepared in various ways. One sample of each pair was exposed for 24 hr to neutrons (10^{13} neutrons/sec/cm²) in the vertical thimble in the Argonne reactor. The other sample of each pair was exposed to intense γ -rays for 3 days in the Argonne Gamma Irradiation Facility (1.42×10^8 r).

This γ -ray source which is composed of fuel elements from the Arco Reactor, provides a γ -ray spectrum like that of the reactor itself. One pair of samples was prepared by evaporation of the solutions (1 ml, 0.5 mc) at room temperature in constricted glass tubes, which were then evacuated and sealed. Another pair of samples was prepared by evaporation in vacuum as just described, but the tubes were filled with air before they were sealed. A third pair of samples consisted of the solutions in tubes filled with air. After exposure to irradiation the tubes were opened, the dried samples were dissolved in water (1 ml), and portions were examined by electrochromatography. Gamma irradiation of the dried samples, like heating, produced some pyrophosphate, diffuse trailing zones and a little material remaining at the starting point. Gamma irradiation of the solution yielded only orthophosphate. Neutron irradiation, which includes intense γ -irradiation, produced much pyrophosphate, diffuse trailing zones and somewhat more material remaining at the starting point. Control experiments showed that preservation of the solution of $\text{H}_3\text{P}^{32}\text{O}_4$ or evaporation of a solution at room temperature followed by preservation of the residue for 3 days did not yield condensation products. These results show conclusively that γ -irradiation alone causes the condensation of $\text{H}_3\text{P}^{32}\text{O}_4$. Radioautographs of the electrochromatograms, reproduced in Figure 40, show that the condensation produced by γ -rays is similar to that produced by heat and by neutrons plus γ -rays.

Summary

These results show that the "carrier-free" tracer recently supplied to us by the Oak Ridge National Laboratory was a mixture of at least two substances in addition to orthophosphate. One of these was pyrophosphate, the other, which does not migrate in the electrical separations, resembles a similar product observed in Oak Ridge phosphoric acid tracer some four years ago, which can also be formed by heating phosphoric acid tracer in a muffle. Both these contaminants are readily removed from the orthophosphate by electrical migration or by sorption in anion exchange columns.

Pyrophosphate is formed rapidly when the tracer solutions are evaporated in open beakers, on a steam bath, or on a hot plate. Pyrophosphate, tripolyphosphate, and a product that does not migrate electrically are formed when the tracer solutions are evaporated in a muffle at temperatures ranging from 150 to 300°. Pyrophosphate and other condensation products are also formed when dried $\text{H}_3\text{P}^{32}\text{O}_4$ is exposed to γ -rays.

These results show that orthophosphate tracer is easily contaminated by condensation of the phosphate. In standard laboratory procedure, this condensation is most likely to result from the action of heat upon the concentrated solutions. It is apparent not only that extreme care must be utilized in the preparation of orthophosphate for use as a tracer in biochemical investigations, but also that the preparation of samples for analysis must be conducted under mild conditions.

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EFFECT OF GAMMA RAYS, OF NEUTRONS PLUS GAMMA RAYS AND OF HEAT ON RADIOACTIVE PHOSPHATE.

Figure 40. Radioautograph illustrating the electrochromatographic examination of $\text{H}_3\text{P}^{32}\text{O}_4$ purified by ion exchange and exposed to γ -rays to neutrons plus γ -rays, and to heat. A shows radioactive phosphoric acid, evaporated to dryness in a vacuum, exposed to neutrons plus γ -rays, and then submitted to migration. B shows a similar sample exposed to γ -rays only. C shows a similar sample sealed in air and exposed to neutrons plus γ -rays. D shows another similar sample sealed in air and exposed to γ -rays only. E shows a fifth sample heated in an open beaker in a muffle at 300° for 0.5 hr.

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PRELIMINARY REPORT: THE OCCURRENCE OF THE ACROSOME IN GRASSHOPPER SPERMATOGENESIS

Rosemarie L. Devine and Theodore N. Tahmisian

The advent of improved techniques in electron microscopy has brought about renewed interest in the Golgi body (including dictyosomes), and this in consequence has stimulated the study of the formation and/or morphology of the acrosome in spermatogenesis.^(1,2,3) It is generally accepted that the acrosome is derived from the Golgi complex (acroblast, idiosome), or, at least, is in close association with it. However, in grasshopper spermatogenesis, only vague and controversial reports of the Golgi body and acrosome are to be found in the literature. Recently, in the course of electron microscope studies of the testis cells of Melanoplus differentialis differentialis Thomas, it was observed that a simple acrosome is formed in the spermatid without any evidence of the presence of a Golgi complex, or of dictyosomes, vacuole or archoplasm. This has prompted an investigation of grasshopper sperm cells to determine the method and locus of formation of the acrosome as well as its morphological characteristics, and to determine whether a Golgi body and/or acroblast is present in a diminutive form which hitherto remains unrecognized.

Preliminary electron microscope studies show a spheroid, osmiophilic proacrosomal body which lies in the cytoplasm close to the nuclear wall in the young spermatid cell. As the spermatid matures and elongates, the cytoplasmic material shifts to the posterior end of the cell. However, the proacrosomal body appears to migrate anteriorly, at the same time differentiating to the acrosome. During this process the acrosome assumes the shape of a cone inverted in relation to the nucleus. The osmiophilic substance of the proacrosomal body is in the cone apex. A less dense substance fills the remainder of the organelle. Eventually the acrosome forms a protuberance at the anterior end of the cell. The acrosomal body is now closely surrounded by one or possibly two discrete membranes lying beneath the cell membrane. Another membrane lies outside the cell membrane, capping the protruding portion of the acrosome and its affiliated parts. It is hoped that further study will clarify the origin of the proacrosomal body, the membranes, and the further differentiation of the acrosome in spermatozoa.

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PROGRESS REPORT: LIQUID SCINTILLATION MEASUREMENTS

Detection of Multiple Tracers by Liquid Scintillation Methods

Walter E. Kisielewski and Frank Smetana

Liquid scintillation counting has achieved some importance as a technique for the measurement of low energy β emitters such as carbon and tritium. The ability of liquid phosphors to respond with excellent sensitivity to low energy β rays can also be applied to higher energy β emitters as well as to γ and α emitters.

Because of the increasing interest in multiple-tracer experiments in biological research, some preliminary measurements were made with a number of different energy β emitters dissolved in a liquid phosphor. Two pairs of radioisotopes have been studied in particular - P^{32} (β , 1.712 Mev) and Ca^{45} (β , 0.254 Mev), both of which are of interest in the study of bone metabolism. The results as shown in Fig. 41 indicate a direct energy response with increasing photomultiplier voltage.

Figure 41. Relative counting efficiency versus photomultiplier voltage. Solid line, P^{32} ; broken line, Ca^{45} .

Scintillating solutions of P^{32} and Ca^{45} were prepared by adding from 0.05 to 0.5 ml of a solution containing the β emitter to 15 ml of absolute alcohol, and adding to this 35 ml of the liquid phosphor (6 mg 2,5-diphenyloxazole per ml of toluene). The characteristics of the individual β emitters were determined by methods previously described.⁽¹⁾

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Figure 41 shows the relative counting efficiency for Ca^{45} and P^{32} at various photomultiplier voltages and at two discriminator windows. At a photomultiplier voltage of 1200 volts, Ca^{45} has an optimum counting efficiency at a 10-100 discriminator window, whereas at a 100- ∞ discriminator window P^{32} has an optimum counting efficiency.

Table 16 shows results of determination on scintillation solutions containing various activity ratios of Ca^{45} and P^{32} . In one channel with a discriminator setting of 10-80 volts, Ca^{45} was measured at 65% efficiency and P^{32} at 14%. In the second channel with a discriminator setting of 80 - ∞ volts P^{32} was measured at 78% efficiency and Ca^{45} at 14%.

The activities of the nuclides under these conditions were calculated by the method described by Hine *et al.*(2)

TABLE 16

Liquid scintillation measurements of mixtures of Ca^{45} and P^{32}

Sample	Nuclide mixture	Sample volume per scintillating solution (ml)	Absolute activity (μc)	Calculated activity (μc)
1	Ca^{45}	.050	.0062	.0060
	P^{32}	.050	.0121	.0124
2	Ca^{45}	.100	.0124	.0116
	P^{32}	.100	.0241	.0242
3	Ca^{45}	.200	.0248	.0235
	P^{32}	.200	.0482	.0477
4	Ca^{45}	.050	.0062	.0054
	P^{32}	.200	.0482	.0482
5	Ca^{45}	.500	.0618	.0603
	P^{32}	.050	.0121	.0121

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PROGRESS REPORT: RADIOELEMENT REMOVAL

Joan F. Fried, Arthur Lindenbaum,
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We are now investigating a series of new chelating agents that have comparatively low affinity for calcium and high affinity for potentially harmful radioactive and non-radioactive metals. These new agents are thus theoretically capable of more effective removal or detoxification of tri- and tetravalent metals than the chelating agents now available.⁽¹⁾ The binding of yttrium by ethylenediaminetetraacetic acid (EDTA), for example, is high ($\log K_f = 17.5$), but the effectiveness of EDTA in removing yttrium from the body is reduced by, among other factors, its affinity for calcium ($\log K_f = 10.7$). In addition, EDTA must be administered as the dicalcium salt to avoid hypocalcemia. Thus a chelating agent with little or no affinity for calcium might be more effective in yttrium removal even though its absolute affinity for yttrium was less than that of EDTA.

One compound now being investigated is Chel 138,* an EDTA analog except that the two symmetrical carboxymethyl groups have been replaced by phenolic groups in which the oxygens act as ligand atoms. Preliminary titration studies indicate that at an approximately equimolar ratio, Chel 138 exhibits a considerable degree of binding for many polyvalent metals at pH 6-9. There is no significant binding of Chel 138 with beryllium. The toxicity and protective action of Chel 138 and other allied compounds are now being investigated, particularly, at present, the effectiveness of Chel 138 in protecting against manganese poisoning. Titration data indicate that the compound binds manganese more efficiently than EDTA.

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*Obtained through the courtesy of the Alrose Chemical Company.
This compound is not available for general distribution.

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PROGRESS REPORT: PYRESIS STUDIES

II. Relationship of Chelating Properties to Antipyretic Action

Marcia White Rosenthal and Joan F. Fried

Salicylic acid and the dye aurintricarboxylic acid (ATA) are alike in possessing hydroxyl groups in the ortho position on benzoic acid. Both are also able to reduce the body temperature of rats with experimentally induced fevers.^(1,2) These findings suggest that the possession of reactive groups capable of metal chelation, and therefore metal inactivation or excretion, may be associated with antipyretic action. In order to test this, a series of compounds, most of which are related to salicylic acid, with and without presumed or known chelating ability, have been tested with respect to their ability to reduce yeast-induced fevers in rats.

The procedure followed in these experiments was that standardized in preceding work.^(1,2,3) Rectal temperatures of adult Sprague-Dawley rats were determined with a Schultheis rapid-recording thermometer, and the rats were sorted at random into groups of five with similar mean temperatures. Fever was then induced by the subcutaneous injection of 3 ml of a freshly-made 15% suspension of dried baker's yeast in 0.85% saline; these animals constituted the fevered rats. About 18 hr later after a temperature reading and when the induced fever was high, they were treated by injection of a test substance and the temperatures were read at intervals thereafter, starting at 1 hr. For each experiment, the controls consisted of fevered and unfevered groups that received injections of saline solution.

Most of the compounds that are chemically related to salicylic acid and that possess potential sites for chelation - gentisic, 4-hydroxyisophthalic, γ -resorcylic, and aurintricarboxylic acids, and probably 5-bromosalicylic acid - were shown to have some degree of antipyretic effect (Table 17). Furthermore, the salicylate-like compounds lacking molecular groups capable of chelation - meta-hydroxybenzoic acid and isophthalic acid - had no significant antipyretic effect. The results are in general consistent with our hypothesis; however, two compounds with chelating groups (sulfosalicylic and ortho-phthalic acids) failed to reduce the fevers.

Although they are not conclusive, the results with this limited series of salicylate-like compounds suggest that their antipyretic effectiveness may depend, at least in part, on their properties as chelating agents. Conclusions beyond this are purely speculative. If chelation is important in antipyresis, certain metals must be involved in the normal mechanisms of temperature regulation, possibly through metal-containing or metal-dependent enzymes. The apparent differences in effectiveness among substances possessing chelating groups, and the lack of effect of two of those tested, may possibly be explained by a number of biological factors related to their size and structural

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TABLE 17

Correlation of molecular structure with antipyresis

Compound	Formula	Presence of chelation site	Dose mg/kg	Rte	ΔT °C*	Reduction below normal**
Salicylic acid (o-hydroxybenzoic acid)		+	125 200 250 125 250 500	i.v. i.v. i.v. i.p. i.p. i.p.	-1.3 -1.4 -1.3 -0.9 -1.4 -2.5	0 0 0 0 + +
m-hydroxybenzoic acid		-	125	i.v.	-0.2	0
Sulfosalicylic acid		+	125 460	i.v. i.p.	-0.3 0	0 0
5-bromosalicylic acid		+	80	i.v.	-0.6	0
4-hydroxyisophthalic acid		+	125 400	i.v. i.p.	-0.1 -2.3	0 +
Phthalic acid (o-benzenedicarboxylic acid)		+	125 400	i.v. i.p.	-0.2 -0.2	0 0
Isophthalic acid (m-benzenedicarboxylic acid)		-	125 400	i.v. i.p.	+0.7 -0.4	0 0
γ -resorcylic acid (2,6 dihydroxybenzoic acid)		+	5 20	i.p. i.p.	-1.3 -3.2	0 +
Gentisic acid (2,5 dihydroxybenzoic acid)		+	125 250 250 400	i.v. i.v. i.p. i.p.	-0.6 -0.2 -0.7 -1.5	0 0 0 0
ATA (aurintricarboxylic acid)		+	125 250	i.v. i.v.	-1.0 -2.0	0 +
Fluorescein sodium		-	125	i.v.	+0.5	0
Acid fuchsin		-	125	i.v.	+0.2	0
EDTA (ethylenediaminetetraacetic acid)		+	600	i.v.	+0.4	0
Aminopyrine		+	25 50	i.v. i.v.	-1.4 -2.9	+ +

*Maximum change in degrees centigrade from febrile controls. This occurred at 1 hr (at the first temperature reading following treatment), except in the case of γ -resorcylic acid when it occurred at 2 hr. All values represent the average of 5 rats.

**Significant reduction of temperature (0.8°C or more) below that of unfevered controls.

differences, e.g., tissue distribution, cellular permeability, and excretion rates. Also important are chemical considerations such as the relative affinity of the chelating compounds for the metal or metals involved in temperature regulation, as well as their relative affinity for these metals compared with other metals in the body.

Two other chelating agents were tried which form somewhat different types of chelates from those formed by the salicylate compounds. The first, aminopyrine, which is used clinically as an antipyretic, was shown to be effective in the present test system. The second, ethylenediaminetetraacetic acid (EDTA), injected in these experiments in the form of the calcium disodium salt, is a well-known chelating agent used for removing calcium as well as foreign metals like plutonium and certain rare earths, from the body. It has never been tested before as an antipyretic, and in these tests showed no such activity. If antipyretic action is associated with chelation, the failure of EDTA in this respect may possibly be ascribed to its failure to reach the site of the metal in the tissues.

Acid fuchsin and sodium fluorescein were selected for testing because they are water soluble dyes resembling ATA structurally, but without chelation sites. Their failure as antipyretics is indirect evidence that the marked effectiveness of ATA is not due to its general size and shape.

It is interesting to note that the intraperitoneal injection of salicylic acid reduced the temperature of fevered rats significantly below that of the unfevered controls. This is in contrast to the experiments in which it was injected intravenously. Further, results reported in the literature indicate that a characteristic of experimental salicylate antipyresis is that temperatures are not reduced below the normal level of the species and this is also true of clinical experience in man.

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