0

ARGONNE NATIONAL LABORATORY
P. O. Box 299
Lemont, Illinois

BIOLOGICAL AND MEDICAL RESEARCH DIVISION
QUARTERLY REPORT

July, August, September, 1956

December, 1956

Preceding Reports:

ANL-5597 - April, May, June, 1956
ANL-5576 - January, February, March, 1956
ANL-5518 - October, November, December, 1955

Operated by The University of Chicago
under
Contract W-31-109-eng-38

DISCLAIMER

This report was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor any agency Thereof, nor any of their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof.

DISCLAIMER

Portions of this document may be illegible in electronic image products. Images are produced from the best available original document.

TABLE OF CONTENTS

	<u>Page</u>
Effects of gamma rays on the serological properties of ovalbumin	
I. Irradiated solutions	
Charles A. Leone and Wendell Landmann	5
II. Irradiated lyophilized protein	
Charles A. Leone and James Vincent.	10
Alteration of the antigenicity of ovalbumin by gamma rays	
Charles A. Leone.	15
Irradiation of poliovirus with gamma rays: a prelude to vaccine production	
Charles A. Leone.	23
The influence of specific nutrients on the mating reaction in <u>Tetrahymena pyriformis</u>	
II. Experiments with complex media	
Howard S. Ducoff	25
III. Enhancement of reactivity by certain reducing agents and by neopyrithiamin	
Howard S. Ducoff	29
On a method of determining association between unordered pairs	
Sylvanus A. Tyler and E. L. Powers.	31
The mean delay in the appearance of the phenotype for phage resistance in <u>E. coli</u>	
Herbert E. Kubitschek and Harold E. Bendigkeit	36
Toxicity of locally deposited yttrium-91 in mice	
I. Experimental design and gross pathology	
Miriam P. Finkel, Hermann Lisco,	
Katherine Lathrop, and Austin M. Brues	39
Progress report: Radiation effects on the <u>Paramecium bursaria</u> mating reaction. Light, ultraviolet and post-ultraviolet light effects on conjugants	
Charles F. Ehret, Antoinette Votava, and	
Lora Harrer	47

TABLE OF CONTENTS

	<u>Page</u>
Purine ribotide - amino acid interactions	
I. Specificity of components of adenosylmethionine-forming reaction in rat liver extracts	
Peter D. Klein.	52
Progress report: Radiation studies in birds. Early changes in plasma glucose concentration following X-irradiation in adult chickens	
S. Phyllis Stearner, Margaret H. Sanderson, and Emily J. Christian	56
Progress report: The biosynthesis of methionine in bacteria. A new transmethylation	
Stanley K. Shapiro	59
Glycogen particles in rat liver homogenates	
A. D. Barton and Anna Kane Laird	61
Progress report: Protein synthesis in pancreas	
III. Subfractionation of microsomes	
Anna Kane Laird and A. D. Barton	65
Organelle patterns of the cell border in <u>Paramecium</u>	
Charles F. Ehret and E. L. Powers	69
Electron microscopic investigation of the wall of the blood capillaries of the liver	
F. Wassermann	71
Progress report: Dose rate effect upon teratogenesis in grasshopper development	
Theodore N. Tahmisian and Betty Jean Wright	75
Progress report: Survival of LAF ₁ mice given daily doses of gamma rays	
George A. Sacher, Douglas Grahn, and Katherine Hamilton.	80
Acute lethal response of first and second generation hybrid mice to whole-body X-irradiation	
Douglas Grahn and Katherine Hamilton.	84

TABLE OF CONTENTS

	<u>Page</u>
Progress report: Deuterium oxide intoxication in rats. Studies on hypophysectomized rats John F. Thomson, Florence J. Klipfel, and Joseph J. Katz.	88
Products of indole-3-acetic acid oxidase from <u>Lupinus albus</u> L. I. Formation of indole-3-carboxaldehyde Robert E. Stutz	91
Mortality of CF No. 1 and RF female mice exposed to single doses of Co ⁶⁰ gamma radiation Howard H. Vogel, Jr.	94
Radiation recovery II. Variation of the time intervals between equal paired doses of Co ⁶⁰ gamma radiation Howard H. Vogel, Jr., Verner Johnson, Nicholas Bink, and Richard Barhorst.	96
III. The rate of recovery from radiation injury as a function of amount of injury John W. Clark, Donn L. Jordan, and Howard H. Vogel, Jr.	99
Mean survival times of neutron-irradiated mice Howard H. Vogel, Jr., John W. Clark, and Donn L. Jordan	102
<u>Pelomyxa palustris</u> from Illinois Edward W. Daniels.	104
3-Amino-1,2,4-triazole I. Effects on radiation lethality Robert N. Feinstein and Sara Berliner	107
II. Effects on tissue catalase: possible similarity to tumor growth Robert N. Feinstein, Sara Berliner, and Frank O. Green.	110
III. Quantitative determination of the compound Frank O. Green and Robert N. Feinstein	118

TABLE OF CONTENTS

	<u>Page</u>
IV. Metabolism in mice	
Robert N. Feinstein and Frank O. Green	122
V. Synthesis of the hydroxy analog	
Frank O. Green	125
Progress report: Studies with chloroleukemia in the rat	
Harvey M. Patt, Eugenia M. Jackson, and	
Mary A. Maloney	127
Progress report: Formation constants for the complexes of	
cobalt and of zinc ions with citric and with oxalic acid	
Edmund L. Lind	129
Progress report: Radioelement removal. II	
Arthur Lindenbaum and Jack Schubert	130
Progress report: Radioelement removal	
III. Manganese removal	
Joan F. Fried, Marcia White Rosenthal, and	
Elizabeth Moretti	131
Progress report: Interactions between divalent transition	
metals and organic anions. II	
Jack Schubert, William Westfall, and	
Arthur Lindenbaum	137
Solubility measurements of CH_4 , N_2 , CO , CO_2 , and C_4F_8 in	
various materials of interest to be used in ionization chambers	
for neutron dosimetry	
S. S. Brar and L. D. Marinelli	138
Serum glutamic oxalacetic transaminase activity as an index of	
liver cell injury due to beryllium poisoning	
Asher J. Finkel and Dorice Czajka	139

EFFECTS OF GAMMA RAYS ON THE SEROLOGICAL PROPERTIES OF OVALBUMIN

I. IRRADIATED SOLUTIONS

Charles Leone, and Wendell Landmann*

From their studies of the effect of α -particles and deuterons on the serological activity of proteins, Pollard and collaborators^(1,2,3) have inferred an all-or-none loss reaction-mechanism. Their serological assay, the precipitin test, was apparently limited to comparisons of single dilutions of the irradiated protein with antinative rabbit serum, in the region of antibody excess.

A more extensive serological study shows that the situation is more complex, at least when γ -rays are used. The effect of treatment with these rays is to produce a variety of modified protein molecules, which exhibit varying degrees of reactivity with antinative serum. This situation greatly complicates the task of describing the serological effect of these rays in quantitative terms. To gain insight into the mechanisms involved, we have used the precipitin reaction over the entire range of combining proportions of antigen and antibody with antisera against ovalbumin.

Crystalline ovalbumin was irradiated in approximately 1% solutions using a 2000-curie Co^{60} source. Ovalbumin irradiated as a solid was subsequently dissolved in 0.01 N NaOH at pH 9 to 10 and readjusted to pH 7 with 0.1 N CH_3COOH ; at the final pH the irradiated albumin was practically completely soluble under the conditions used.

Aerated 0.90 to 0.95% solutions of ovalbumin, adjusted to pH 7.2 with 0.01 N NaOH and 0.1 N CH_3COOH were irradiated to receive various γ -ray dosages, expressed in terms of the energy in electron volts absorbed in the system per protein molecule (ev/mol). Figures 1 and 2 are plots of the serological activity of several of these irradiated solutions as compared to that of native ovalbumin. The abscissas give the concentration of the antigen as established from Kjeldahl nitrogen determinations, using the 6.25 protein conversion factor of native ovalbumin. Ordinates give the amount of turbidity measured at each antigen-antibody combining proportion, and the corresponding number of micrograms of nitrogen precipitated in each mixture of antigen-antibody. A volume of 0.3 ml of antinative serum was added to 1.7 ml of each antigen concentration. The reaction-time adopted as standard for all serological tests was 24 hr at room temperatures (23-24° C).

Figure 1 is an arithmetic plot of the precipitin curves and emphasizes the loss of activity in the region of antibody excess among the irradiated solutions as the γ -ray dosage is increased. A simple explanation of these data is that the irradiations destroyed the ovalbumin directly as the dose was increased.

*From the Chemistry Division.

Figure 1. Precipitin curves of native and irradiated ovalbumin preparations vs. antinative ovalbumin. Arithmetic plot showing loss of reactivity with increasing γ -ray dosage. Ovalbumin preparations were irradiated as 0.9% aerated solutions at pH 7.2. γ -ray dosages are expressed as electron volts (ev) of absorbed energy per protein molecule.

Figure 2.

Precipitin curves of native and irradiated ovalbumin preparations vs. antinative ovalbumin. Geometric plot showing shift toward region of high antigen concentration and change in form of curves with increasing γ -ray dosage. Same conditions of irradiation as in Figure 1. The 1.0 μ g and 2.1 μ g levels of precipitated nitrogen were utilized in establishing the plots in Figure 3.

Figure 2 is a logarithmic plot of the same precipitin curves presented in Figure 1, and this presentation of the data requires a more extensive explanation of the alterations in serological activity induced by irradiations. If the effect of the radiation on the protein had been an all-or-none loss reaction then the precipitin curves for the irradiated systems should be like the native, except that, as the protein was increasingly destroyed under increasing dosages of radiation, the curves should shift along the abscissa in the direction of higher protein concentration. If a curve had shifted by a factor of two, it would have meant that half of the antigen molecules had been completely destroyed. Shifts along the abscissa do occur, but the observed precipitin curve of each irradiated solution differs from the native curve in at least two respects. The height of the maximum is different and the curve is broader. With increasing dosages of irradiation, these characteristics become more pronounced. The differences between the native and irradiated systems are interpreted to mean that the latter contain a heterogeneous population of molecules exhibiting various degrees of serological reactivity with antinative serum.

A remarkable phenomenon is the appearance of increased precipitation at the maxima of irradiated solutions of ovalbumin which received moderate radiation dosages. The causes for this departure from the native reaction-curve reside in the moderately labile portion of the irradiated system. One possibility is that it is the result of a slight loosening or unfolding of the molecular configuration. Another possibility is that it is connected with the dimerization of the irradiated protein. The presence of dimers in these irradiated solutions has been shown by our preliminary analyses using ultracentrifugation. Polymerized ovalbumin would require less antibody to be precipitated and would have a different antigen-to-antibody ratio at the zone of optimal combining proportions than ordinary native protein. Precipitating antibodies would then produce a greater mass of precipitate per molecule, and, in the serological tests as performed in these experiments, would cause a slight shift of the curve toward the region of higher antigen concentration.

To obtain a complete description of the effect of radiation on a protein, in terms of the precipitin test, we should be able to calculate G values, i. e., the relative amount of loss of protein in unit terms of 100 ev of absorbed energy per molecule, for the various serologically active protein species in an irradiated system; we should also be able to present the precipitin curves as they would have been observed if these various species were tested separately. Some light can be thrown on this problem since it is possible to fractionate the irradiated systems; the technique is based on the fact that the modified protein molecules have decreased stability, as characterized by their increased thermal lability and their insolubility at the isoelectric point (pH 4.85) of normally soluble native ovalbumin. The serological activity of fractions thus obtained has convinced us that the irradiated systems contain a more or less continuous spectrum of molecular species with serological activities ranging from high to zero.

If we tentatively assumed that the serological activity of an irradiated solution was due to only one serologically active molecular species, the activity of this constituent would be given by the height of the maximum. The relative concentration of these molecules would be given by the horizontal distance of the curve for the irradiated system from the curve for the native system. Since, however, irradiation alters the form of the curve this horizontal distance cannot be determined unequivocally. If, as is shown in Figure 3, we compare horizontal distance between maxima, we get one result. If we compare points of equal precipitation values we get other results, which depend upon the values chosen. Nevertheless, the different "dosage" curves obtained by using several such methods of comparison do agree as to order of magnitude. The $D_{37\%}$, i.e., the γ -ray dose required to reduce the serological activity to 37% of its original value, for the different curves of Figure 3 lies in the range of 80 to 250 ev per protein molecule, hence the G value for the formation of relatively inactive protein molecules is of the order of unity. Just how meaningful this calculation is remains to be seen.

Figure 3. Serological activity of irradiated, aerated, 0.9% ovalbumin solutions, pH 7.2, in relation to absorbed energy expressed as ev/molecule. The points "constant precipitate 1.0 μ g/ml" and "2.1 μ g/ml" were obtained from Figure 2. Curve X represents the nondenatured protein in the irradiated systems.

As far as could be discovered by precipitation with trichloroacetic acid no appreciable fragmentation occurs in proteins irradiated as 1% solutions. There is an appreciable amount of denatured material however, as revealed by precipitation at the isoelectric point. Curve X (Figure 3) depicts the nondenatured protein in the irradiated systems under consideration.

References

1. Pollard, E. Primary ionization as a test of molecular organization. *Advances in Biol. and Med. Phys.* 3:153-189 (1953).
2. Huber, W. Cold sterilization by electron beam as a possible tool for the inactivation of the virus of homologous serum jaundice in plasma. *Ann. N. Y. Acad. Sci.* 55:536-542 (1952).
3. Pollard, E. C., and L. M. Kraft. Inactivation of MEF1 poliomyelitis by ionizing radiation. *Proc. Soc. Exptl. Biol. Med.* 88:331-333 (1955).

EFFECTS OF GAMMA RAYS ON THE SEROLOGICAL PROPERTIES OF OVALBUMIN

II. IRRADIATED LYOPHILIZED PROTEIN

Charles A. Leone, and James Vincent*

Ovalbumin, irradiated as a dry solid, is altered directly by the ionization processes which occur within the molecule. Structural changes in the protein molecules can be minor or drastic depending upon the location and number of ionization events which occur. The effect of such treatment with γ -rays, as was true for irradiated solutions,⁽¹⁾ is to produce a conglomeration of molecules representing an entire spectrum of modifications from the native type. Serological analyses were made on irradiated ovalbumins as a means of studying the mechanisms of the structural degeneration of the protein molecules under the influence of γ -rays.

Dry ovalbumin preparations, twice recrystallized, were irradiated at 0° to 4°C under vacuum in a high intensity (1.5×10^6 rep/hr) uniform field of γ -rays. Following irradiation the proteins were dissolved in a dilute solution of NaOH (pH 10) and then adjusted with 0.1 N acetic acid to pH 7.2.

Figures 4 and 5 are representations of the serological activities of the irradiated proteins with antinative sera. Figure 4 is an arithmetic plot of the precipitin curves and Figure 5 is a logarithmic plot of the same data. As the γ -ray dosages increase, there is a loss of serologically active protein which causes a decrease in the amount of precipitate formed in the region of antibody excess (Figure 4) and a shift of the curves toward the region of higher antigen concentration (Figure 5). The depression of the peaks and the broadening of the curves approximate the results obtained with irradiated solutions. The marked rise in the peaks of the curves obtained with some of the low-dosage, irradiated, 1% solutions⁽¹⁾ was not so noticeable in the irradiated solids. The significance of this variation is not understood. The lateral shifts of the curves obtained for irradiated solids is far greater than those obtained for irradiated solutions. This latter difference provides evidence that the ionization events which occur within the lyophilized protein molecules during irradiation cause major structural changes, and that the indirect effects of the ionized, water medium in irradiated 1% solutions is relatively minor.

Figure 6 is a representation of several types of analyses performed with the data given in Figures 4 and 5. Comparisons among equivalent amounts of precipitate (constant ordinate) in the region of antibody excess, or of the amount of precipitate developed at a given concentration of ovalbumin (constant abscissa) or of ratios between the maxima of the curves

*From the Chemistry Division (Student Aid from the University of Redlands).

Figure 4. Precipitin curves of native and irradiated ovalbumin preparations vs. antinative ovalbumin. Ovalbumin preparations were irradiated under vacuum as lyophilized proteins. γ -ray dosages are expressed as electron volts (ev) of absorbed energy per protein molecule. This is an arithmetic plot showing loss of reactivity with increasing γ -ray dosage.

Figure 5. Precipitin curves of native and irradiated ovalbumin preparations vs. antinative ovalbumin. Ovalbumin preparations were irradiated under vacuum as lyophilized proteins. γ -ray dosages are expressed as ev/protein molecule. Geometric plot showing shift of curves toward region of high antigen concentration, and change in form, with increasing γ -ray dosage. $\bullet$, native ovalbumin; $\circ$, ovalbumin irradiated with 63 ev/molecule; Δ , 140 ev/molecule; $\square$, 300 ev/molecule; $\odot$, 478 ev/molecule; $\bullet$, 878 ev/molecule.

and the constant ordinate or constant abscissa, all yield similar dosage curves. $D_{37\%}$ varies in the different curves between 90 and 110 ev/mol, i.e. the G value for the rate of formation of serologically inactive protein is of the order of unity. If it is assumed that the preparations contained only one serologically active constituent, the sensitive volume for the formation of inactive protein per primary ionization process is the whole protein molecule.

Curve Δ (Figure 6) represents the non-denatured protein portion, obtained by subtracting both (a) the low-molecular-weight split products by precipitating with trichloroacetic acid and measuring the Kjeldahl nitrogen in the supernatant, and (b) the incipient denatured protein precipitable at pH 4.85. This curve lies below, but is in the same general position as the curves which represent the loss of serological activity. There is not necessarily an inconsistency here, since protein denatured by γ -rays under the conditions described exhibits considerable reactivity with antinative serum. Figure 7 gives an example of the serological behavior of an irradiated preparation of ovalbumin before and after the denatured fraction was removed. Shown also is the serological behavior of the denatured protein, prepared by separating the pH 4.85 precipitate, redissolving it at pH 9 and then readjusting to pH 7.

With dosages below 100 ev/mol the irradiated protein is completely soluble between pH 7 and 9 at 0°C, and the serological properties studied are independent of the pH within this range. More strongly irradiated protein requires higher concentrations of NaOH (pH 9-11) to obtain dissolution. The serological data obtained from the latter solutions reflect the influence of pH and the time used in preparing them and are not strictly comparable with those of the former, lower pH solutions. After dissolution all preparations were brought to pH 7.2 where they were clear and sufficiently stable for serological study.

Heat-denatured ovalbumin, prepared by heating native protein for 2 hr at 70°C, pH 4.85 and dissolving the resulting precipitate in dilute NaOH at pH 10, as was done with radiation-denatured ovalbumin, has virtually no reactivity with antinative sera over a broad range of protein concentrations. It appears from these observations that the radiation-denatured protein molecule is in a structural state more like the native than is the heat-denatured protein. Optical activity studies lead to the same conclusion. The specific rotations for the radiation-denatured and heat-denatured proteins which were compared serologically above were 68° and 76° respectively, compared to 38° for the native, all measurements being made at pH 7.2, 0°C., 5410 Å.

Solutions of irradiated ovalbumin contain low-molecular-weight fragments sufficient to account for as much as 10% of the total nitrogen present in the system. Present also is a large quantity of thermolabile

Figure 6. Serological activity of irradiated ovalbumins as shown by several methods. □, maximum precipitate (M); ○, relative amount of antigen (A) for 1.33 g N₂ per ml; ◇, A/M; x, relative amount of precipitate (P) from 3 μg of antigen/ml; *, P/M; Δ, the protein soluble at pH 4.85. (Δ represents the non-denatured protein portion of the irradiated preparations.)

Figure 7. Precipitin curves of native and irradiated ovalbumin and of fractions derived from irradiated ovalbumin (An-Ab). The sample was irradiated under vacuum as lyophilized protein, and absorbed 32.4 ev/protein molecule.

proteins which precipitate when the system is heated for two hours at 50°C., pH 4.85. The remainder of the proteins are stable and remain in solution at the isoelectric point. Studies are currently under way which are designed to reveal the exact nature of the low-molecular-weight fragments, and to do a finer "denaturation-fractionation" of the thermolabile components, so that an analysis of their serological correspondence with native ovalbumin can be completed, and some determinations can be made of the chemical nature of the structural damage caused by ionizing radiations.

Reference

1. Leone, C. A., H. Fricke, and W. Landmann. Effects of gamma rays on serological properties of albumin. I. Irradiated solutions. This report, p. 5.

ALTERATION OF THE ANTIGENICITY OF OVALBUMIN BY GAMMA RAYS

Charles A. Leone

Vaccine preparation from viruses by means of high frequency ionizing irradiation is of great current interest. Studies on the effects of γ -rays on serological properties of ovalbumin were performed as a necessary prerequisite to experiments involving infective viruses. Reported here are some experiments on changes observed in the antigenicity of irradiated ovalbumins as indicated by their capacity to sensitize guinea pigs to native ovalbumin.

Twice recrystallized ovalbumin, either in the powdered state or in approximately 1% solution, was irradiated using γ -rays from a 2000-curie Co^{60} source. Samples were characterized or designated in terms of the electron volts absorbed into the system per protein molecule (ev/mol). For convenience of the reader, Figure 8 describes the relationship between ev/mol and roentgen-equivalent-physical (rep) for 1% solutions and lyophilized preparations of irradiated ovalbumin.

Native ovalbumin solutions were prepared by dissolving lyophilized protein in water adjusted to pH 7.2 with 0.01 N NaOH. Aliquots of these solutions were irradiated at 0 to 4°C. Ovalbumin, irradiated as lyophilized protein under vacuum and at 0 to 4°C., was subsequently dissolved in water adjusted with the dilute alkali solution to pH 9-10, and then readjusted to pH 7.2 with 0.1 N CH_3COOH .

Figure 8. Relationship between roentgen equivalent physical (rep) and electron volts (ev) per molecule ovalbumin. Values for 1% solutions are considered to be approximately the same as for water and all of the absorbed energy is assumed to be transferred to the protein molecules. Values for lyophilized, solid ovalbumin are actually 4% less than those indicated, because the protein has 4% less hydrogen than water. Absorptions of energy are calculated on the basis of conversion of Fe^{++} to Fe^{+++} in a solution of 0.001 N FeSO_4 in 0.8 N H_2SO_4 and 0.001 N NaCl.

Hartley strain guinea pigs, 4 weeks old, weighing 225 to 325 g, were sensitized by injecting, intra-abdominally, known amounts of ovalbumin in a 0.5-ml volume. The amounts of antigen used to sensitize the guinea pigs ranged from 0.001 to 30 μ g. For each preparation of irradiated ovalbumin 5 or 6 concentrations of protein, in a series of 3-fold dilutions, were each injected into a group of 12 guinea pigs; hence, for each sample 60 or 72 animals were sensitized.

On the 28th day after the day of sensitization, the guinea pigs were challenged with 1 mg of native ovalbumin in a volume of 0.5 ml. In order to be sure that the challenging dose was injected in the circulating blood, the accessory cephalic vein of a foreleg was exposed by snipping away a patch of skin with scissors and the needle was placed directly in the vein.

In following the course of the anaphylactic shock, seven symptomatic categories were arbitrarily established:

1. No detectable effect.
2. Reddening of the pinna of the ears and raising the hair on the scruff of the neck.
3. Pursing the lips and rubbing the nose with forepaws.
4. Coughing.
5. Labored breathing.
6. Convulsions.
7. Death.

The guinea pigs usually developed the symptoms in the sequence given. If an animal did not begin to respond by the end of 3 min no symptoms developed, although all surviving animals were also observed at 30 min and 1 hr. Some of the animals that responded did not die. Although careful records were kept of the progress of the anaphylactic shock in each guinea pig, Tables 1 and 2, which summarize the results obtained, indicate only those animals killed, or responding, (this class includes the above categories from two through six). By subtracting the number responding from the total number receiving a given sensitizing dose, the number showing no effect can be obtained. Each series of tests included four groups of guinea pigs that received experimental (irradiated) proteins and, in order to provide an adequate control, a group of guinea pigs that received native protein.

Figure 9 is a graphic representation of the relative capacities of irradiated, 1% solutions of ovalbumins and native ovalbumin to sensitize guinea pigs to a minimal challenging dose of native ovalbumin.

Most striking is the fact that the solutions which absorbed 305-337 ev/molecule of protein had a greatly augmented capacity to sensitize against native proteins. The 50% sensitizing dose SD_{50} (for

TABLE 1

Anaphylactic deaths and responses of guinea pigs to irradiated ovalbumins

Group	Condition of protein at irradiation	γ -ray dose, ev/mol	Fraction* killed or responding											
				Sensitizing dose of ovalbumin, mg										
				0.12	0.24	0.37	0.74	1.1	2.2	3.3	6.6	10	20	30
N ₁ **	-	0	K†	0/12	-	0/12	-	4/12	-	6/12	-	10/12	-	-
			R†	0/12	-	2/12	-	5/12	-	7/12	-	11/12	-	-
I	1% Soln.	337	K	11/12	-	8/12	-	7/12	-	10/12	-	10/12	-	-
			R	12/12	-	12/12	-	11/12	-	12/12	-	11/12	-	-
II	1% Soln.	140	K	2/12	-	3/11	-	10/12	-	7/12	-	10/12	-	-
			R	2/12	-	4/11	-	10/12	-	9/12	-	11/12	-	-
III	1% Soln.	4242	K	0/12	-	2/12	-	4/12	-	1/11	-	1/11	-	-
			R	0/12	-	3/12	-	4/12	-	2/11	-	3/11	-	-
D	Dry	?	K	-	0/12	-	2/12	-	2/12	-	4/12	-	3/12	-
			R	-	0/12	-	3/12	-	3/12	-	7/12	-	8/12	-
N ₂	-	0	K	-	-	0/12	-	1/12	-	3/12	-	6/12	-	-
			R	-	-	0/12	-	3/12	-	4/12	-	7/12	-	-
IV	1% Soln.	305	K	1/16	5/15	-	8/16	-	12/16	-	16/16	-	-	-
			R	2/16	8/16	-	12/16	-	14/16	-	16/16	-	-	-
V	1% Soln.	620	K	0/12	-	0/12	-	1/12	-	4/12	-	-	-	-
			R	0/12	-	0/12	-	3/12	-	8/12	-	-	-	-
VI	1% Soln.	3890	K	-	0/12	-	0/12	-	0/12	-	1/12	-	2/12	-
			R	-	0/12	-	0/12	-	0/12	-	1/12	-	4/12	-
VII	1% Soln.	32.4	K	-	-	0/12	1/12	-	3/12	-	5/12	-	7/12	-
			R	-	-	0/12	1/12	-	5/12	-	8/12	-	9/12	-
N ₃	-	0	K	0/12	-	0/12	-	1/12	-	4/12	-	6/12	-	9/12
			R	0/12	-	1/12	-	3/12	-	6/12	-	9/12	-	11/12
VIII	Dry	75	K	0/12	-	0/12	-	1/11	-	1/12	-	5/12	-	-
			R	-	-	0/12	-	1/11	-	3/12	-	7/12	-	-
IX	Dry	150	K	0/12	-	0/12	-	0/12	-	6/12	-	6/12	-	-
			R	-	-	0/12	-	1/12	-	6/12	-	8/12	-	-
X	Dry	306	K	0/12	-	0/12	-	0/12	-	2/12	-	1/12	-	-
			R	0/12	-	0/12	-	0/12	-	7/12	-	3/12	-	-
XI	Dry	570	K	-	0/12	-	0/12	-	0/12	-	1/12	-	-	-
			R	-	0/12	-	1/12	-	3/12	-	3/12	-	-	-
XII	Dry	878	K	-	-	0/12	-	1/12	-	1/12	-	0/12	-	2/12
			R	-	-	0/12	-	1/12	-	2/12	-	6/12	-	7/12
XIV	1% Soln.	306	K	0/12	-	1/12	-	4/12	-	6/12	-	11/12	-	12/12
			R	1/12	-	4/12	-	10/12	-	11/12	-	12/12	-	12/12
N ₄	-	0	K	-	-	0/9	-	0/12	-	1/11	-	3/12	-	9/11
			R	-	-	1/9	-	4/12	-	8/11	-	10/12	-	10/11
XVIII	1% Soln.	336	K	-	-	0/10	-	1/12	-	3/11	-	9/12	-	8/11
			R	-	-	4/10	-	10/12	-	9/11	-	10/12	-	10/11
XX	Dry	60	K	-	-	0/12	-	0/9	-	2/9	-	5/12	-	7/9
			R	-	-	2/12	-	2/9	-	5/9	-	10/12	-	9/9
XXI	Dry	120	K	-	-	0/9	-	0/12	-	3/12	-	5/10	-	9/11
			R	-	-	2/9	-	4/12	-	10/12	-	7/10	-	11/11
XXII	Dry	300	K	-	-	0/11	-	0/11	-	0/10	-	1/12	-	4/10
			R	-	-	0/11	-	1/11	-	0/10	-	5/12	-	7/10

*F = the number of guinea pigs killed (K) or responding (R) of the total number receiving a given sensitizing dose, and surviving until the time at which they were challenged.

**N = native ovalbumin.

†k = killed.

†R = anaphylactic responses, but no death within 1 hour's observation.

TABLE 2

Anaphylactic killings and responses of guinea pigs to fractionated irradiated ovalbumins

Group	Condition of protein at irradiation	γ -ray dose, ev/mol	Ovalbumin fraction	Fraction* killed or responding					
				Sensitizing dose of ovalbumin, mg					
				0.12	0.37	1.1	3.3	10	30
N ₅ **	-	-	K† Native	0/12	2/12	6/11	4/12	4/12	12/12
			R‡	0/12	4/12	6/11	7/12	9/12	12/12
XXIV	-	324	K { Supernatant of whole-	1/12	1/12	7/12	11/12	12/12	-
			R { irradiated	4/12	9/12	10/12	12/12	12/12	-
XXV	-	324	K { Heat precipitate, 52°C	0/12	1/12	4/12	6/12	11/12	-
			R { 1-1/2 hr	0/12	2/12	5/12	8/12	11/12	-
XXVI	-	324	K { Supernatant of heat-	-	4/12	4/12	8/12	11/12	12/12
			R { labile fraction	-	5/12	5/12	9/12	10/12	12/12
XXVII	1% soln.	324	K Irradiated solution	2/12	4/12	9/12	9/12	10/12	11/12
			R	4/12	7/12	10/12	10/12	11/12	11/12

*Fraction = the number of guinea pigs killed (K) or responding (R) of the total number receiving a given sensitizing dose, and surviving until they were challenged.

**N = native ovalbumin.

†K = killed.

‡R = anaphylactic responses but no death within one hour's observation.

Figure 9. Anaphylactic responses of guinea pigs to irradiated, aerated 0.9% solutions of ovalbumin at pH 7.2. Solutions are identified in terms of electron volts (ev) absorbed per protein molecule.

any type of anaphylactic response) for native ovalbumin was approximately $2.5 \mu\text{g}$. The SD_{50} for the 305-337 ev/molecule preparations of ovalbumin was approximately $0.1 \mu\text{g}$, a 25-fold increase in capacity to sensitize to native protein. The solution receiving 140 ev/molecule had a slightly augmented capacity to sensitize to native protein. At 620 ev/molecule the sensitizing properties of an irradiated solution of ovalbumin were approximately equal to native protein. At higher γ -ray dosages the irradiated solutions had progressively lowered capacities to sensitize guinea pigs.

If only the data obtained with the 305-337 ev/molecule solutions and native solutions are considered and the deaths following anaphylactic shock are compared, the 50% lethal dose (LD_{50}) for native protein is $6.3 \mu\text{g}$ and that for irradiated protein is $1.3 \mu\text{g}$ and that for irradiated protein is $1.3 \mu\text{g}$ (Figure 10).

The capacities of irradiated, 1% solutions of ovalbumin to sensitize guinea pigs, as compared to the capacities of the native protein, are presented in Figure 11.

The anaphylactic responses of guinea pigs to ovalbumin which had been irradiated under vacuum in the lyophilized state, and subsequently dissolved in dilute alkaline solutions, are presented in Figure 12. Augmentation in antigenicity was not obtained with any of these preparations. Preparations absorbing between 60 and 306 ev/molecule had sensitizing capacities within the normal range of variation for native ovalbumin. Plots of the data obtained with the individual preparations within the above range were, in general, slightly below the average curve for native ovalbumin. An average curve representing these dry, irradiated ovalbumins is given in the curve designated as 175 ev/molecule (Figure 12). The irradiated preparations absorbing between 570 and 878 ev/molecule had definitely lowered capacities to sensitize guinea pigs to a challenging dose of native ovalbumin. An average curve representing the data obtained with preparations absorbing the higher γ -ray dosages is designated as the 724 ev/molecule curve in Figure 12.

A plot of the relative effectiveness of ovalbumins irradiated under vacuum as lyophilized protein, is presented in Figure 13.

No adequate explanation can be offered, as yet, of the specific changes that are induced in the protein molecules of the irradiated solutions and that increase their antigenicity in guinea pigs. As a preliminary, we have established by means of ultracentrifugal analysis that dimers (and perhaps trimers) were present in solutions which absorbed 300 ev/protein molecule. The relative proportion of the polymerized molecules to monomers was small. Should the observed effects on the guinea pigs be due to the polymerization of ovalbumins, then because of their relatively few numbers the polymers are all the more remarkable as antigens capable of inducing the formation of antibodies to native protein.

Figure 10. Anaphylactic deaths of guinea pigs sensitized to irradiated 1% ovalbumin solutions which absorbed between 305 and 337 ev per molecule.

Figure 12. Anaphylactic responses of guinea pigs to irradiated, solid ovalbumin. Preparations are identified in terms of absorbed γ -ray dosage as ev/protein molecule.

Figure 11. Relative sensitivity of guinea pigs to irradiated, aerated 0.9% ovalbumin solutions at pH 7.2.

Figure 13. Relative sensitivity of guinea pigs to irradiated, solid ovalbumin.

The loss of antigenicity in solutions of ovalbumin absorbing the larger doses of γ -rays is not due to the destruction of protein. Both Kjeldahl determinations for protein-nitrogen and ultracentrifugal runs on solutions absorbing as much as 4242 ev/molecule indicated that the original amount of protein was still present.

The loss of antigenicity in the lyophilized, irradiated protein can readily be credited to the actual destruction of protein. Biuret analysis of the solutions derived from the irradiated solids at pH 9-10 yielded increasing quantities of peptides as the γ -ray dosages increase. As much as 35% of the nitrogen available in a preparation of irradiated, dry protein was in non-protein.

In an attempt to establish the fraction of an irradiated solution which was responsible for the increased antigenicity a sample absorbing 324 ev/molecule of protein was fractionated into components. The pH of a portion of original, irradiated solution (I) at 0° C, was adjusted with 0.1 N CH_3COOH from pH 7.2 to pH 4.85. Almost no radiation-denatured protein was precipitated, but the solution was centrifuged at 2000 rpm and the supernatant was designated as fraction S_1 . After an aliquot of S_1 was heated at 50°C for 1-1/2 hr, a heavy precipitate formed. After centrifugation at 2000 rpm, the heat-stable supernatant was designated S_2 . Both S_1 and S_2 were adjusted with 0.1 N NaOH to pH 7.2. The heat-labile precipitate was redissolved in dilute NaOH at pH 9, adjusted with acetic acid to pH 7.2 and designated P_2 . A comparative study was then made in guinea pigs of the relative capacities of the preparations I, S_1 , S_2 , and P_2 , and a native preparation N. Table 2 presents the data obtained from these experiments. Figure 14 is a graphic representation of these same data.

The irradiated solution I and fraction S_1 possessed increased antigenicity. The heat-stable S_2 resembled N, the native ovalbumin. The heat-labile component P_2 which evidently conferred the increased antigenicity on I and S_1 , did not itself have increased antigenicity following the heating at 50°C for 1-1/2 hrs. The structural degradation caused by the heating and subsequent dissolution at pH 9 had evidently removed the capacity of P_2 to exceed the native as a sensitizing antigen.

Figure 14. Anaphylactic responses of guinea pigs to ovalbumin fractions derived from a 1% solution irradiated with γ -rays to absorb 324 ev/molecule. ●, native albumin (N); ○, irradiated solution I; ⊙, supernatant portion of whole irradiated solution (at pH 4.85, 0°C.) (S_1); Δ, heat-labile precipitate (52°C. for 1-1/2 hr) (P_2); ⊙, heat-stable fraction (S_2); —, possible position of ⊙ curve.

IRRADIATION OF POLIOVIRUS WITH GAMMA RAYS: A PRELUDE TO VACCINE PRODUCTION

Charles A. Leone

A series of studies was undertaken which were designed to explore the feasibility of producing a poliovaccine by the use of high energy γ -rays. A preliminary observation by the author that, under certain conditions, irradiated solutions of ovalbumin actually had a greatly increased capacity to induce antibodies against native protein, suggested that a similar finding might be possible with viruses. Poliovirus offered additional promise in that the ratio of infectious doses to physical particles of virus per unit volume is estimated to be as high as 1:2000. The relationship between infectious doses and physical particles is not well understood. The antigenic potentiation of cultures of poliovirus by γ -ray irradiation is one of the objectives of this program. As necessary preludes to the practical problems of production of virus vaccines, a scientific study of the effects of irradiation on the tissue culture infectivity and serological potency of poliovirus under a variety of experimental conditions is under way.

Type III poliovirus in neutral Tissue Culture Medium No. 199 (TC 199) containing 2×10^6 infectious doses per ml was provided for irradiation by Eli Lilly and Company, Indianapolis, Indiana. Type III poliovirus, suspended in neutral 1% sodium phosphate plus 0.025% sodium chloride, containing 10^7 infectious doses per ml was provided by Merck and Company, West Point, Pennsylvania. Complete safety tests and a limited number of potency tests were performed on the irradiated samples by the respective pharmaceutical companies.

The virus preparations were flame-sealed in glass ampoules, and, as a safety precaution, further sealed in specially designed leak-proof brass cans. In transit between this laboratory and the pharmaceutical companies, during temporary storage, and during irradiation, the virus preparations were kept between 0°C and 4°C . The data herein reported are for irradiations of virus materials suspended either in TC 199 or dilute salt solution, as received from the pharmaceutical companies.

All irradiations were performed in the Argonne High Gamma Facility in a uniform γ -ray field of high intensity (approximately 1.5×10^6 rep/hr).

Figure 15 is a graphic representation of the effects of γ -rays upon the tissue culture infectivity of Type III poliovirus suspended in either TC 199 or dilute salt solution. In TC 199 approximately 9.2×10^6 rep were required to obtain preparations which were sterile. In the dilute salt solution sterility

was attained at 1×10^6 rep. Preliminary tests on potency indicate that antigenicity is destroyed by the time sterility is acquired. Potency tests were performed in mice, guinea pigs and monkeys, and by complement fixation tests.

Figure 15. Sterilization of poliomyelitis virus Type III by γ -irradiation, as revealed by tissue culture titration. Irradiated in solution at 4°C , pH 7.0. TC 199 is Tissue Culture Medium 199 (Difco). Saline is 1% phosphate buffers plus 0.025% NaCl.

Our potency tests, thus far, indicate that the sensitive volume for loss-of-potency, as compared to the sensitive volume (10^7 A^3) for loss-of-infectivity, may be relatively larger than that found to be usual in other types of viruses. The ratio of these two sensitive volumes is typically of the order of 1/100, and is as high as one could accept if radiation is to be useful in the preparation of poliovaccine. Particular attention in future experiments will be focused on establishing conditions under which the potency loss is as small as possible compared to loss of infectivity. Characteristics which need to be varied during irradiation are virus concentration, pH, oxygen pressure, inorganic and organic addition substances, temperature, physical state (fluid, frozen) and hydrostatic pressure.

PROGRESS REPORT: THE INFLUENCE OF SPECIFIC NUTRIENTS
ON THE MATING REACTION IN TETRAHYMENA PYRIFORMIS

II. Experiments with complex media

Howard S. Ducoff

It was previously reported⁽¹⁾ that additions of purines and pyrimidines, or of glucose, did not greatly affect conjugation in washed aqueous suspensions of *Tetrahymena* (strains LF and Ham-3 of Austin Phelps) grown in proteose peptone, but that a salts-buffer-amino-acid mixture suppressed the reaction. Results in this type of experiment were difficult to evaluate, however, because conjugation in the controls was usually late (40 to 60 hr after mixing) and often involved only a small proportion of the animals used.

An attempt was then made to investigate the possible elaboration by separate strains, or by mixtures, of "hormone-like" materials which would accelerate conjugation after the mixing of washed animals. The media tested were: supernatant portions of proteose peptone cultures, or of aqueous suspensions of each strain and of a mixture, and residues of the aqueous suspensions after centrifugation $4\frac{1}{2}$ hr later. Each preparation was frozen and thawed several times to eliminate any living animals. Water and fresh proteose peptone were used as controls. Freshly washed suspensions of LF animals were added to the test media in depression plates, and 16 hr later, similarly washed Ham-3 animals were added. Up to this point, all operations were performed aseptically. The various mixtures were examined at intervals, and conjugation was scored according to the scheme shown in Table 3.

TABLE 3

Scoring System

0	no conjugation
II	one or two pairs seen
III	occasional; several pairs, but not more than one pair per field
IV	frequent; one to several pairs per field
V	5 to 15% of the organisms seen are conjugating pairs
VI	15 to 30% of the organisms seen are conjugating pairs
VII	more than $1/3$ of the organisms seen are conjugating pairs

Results of this experiment, as given in Table 4, reveal two important points. First, it is possible to obtain conjugation even in axenic culture media which, while partially exhausted, were still capable of supporting proliferation (columns 3 and 4). Secondly, the greatly enhanced conjugation in water in which animals had been previously incubated cannot be ascribed to hormone-like substances in the fluid; enhancement was obtained with water previously used to suspend LF animals for only $4\frac{1}{2}$ hr, as well as with water in which Ham-3 animals or mixtures had been similarly suspended, but not when the LF test animals were incubated in fresh water for 16 hr before the Ham-3 animals were added. It would appear, rather, that materials released by the freezing and thawing of animals in the supernatants may have favored conjugation. This interpretation is supported by the observation that experiments in which animals were centrifuged very gently yielded poorer reactions than experiments in which rough handling damaged a significant number of the ciliates.

TABLE 4

Effect of previous conditioning of the suspending medium

Hours after mixing (1)	Proteose peptone			Water			Residue		Water
	Fresh (2)	LF (3)	Ham (4)	LF* (5)	Ham* (6)	Mixt.* (7)	Ham (8)	Mixt. (9)	Fresh (10)
24	0	0	0	VII	VII	VI	VII	VII	0
28	0	0	0	VII	VII	VI	VII	VII	0
31	0	0	0	VII	VII	VI	VII	VII	0
53	0	VI	IV	III	IV	0	III	0	0

*After $4\frac{1}{2}$ hr incubation.

Accordingly, washed suspensions of animals grown in proteose peptone were mixed in depressions containing water, dilute yeast extract, dilute liver extract, peptone, or proteose peptone. Conjugation was greatly enhanced in the presence of yeast extract, and to a lesser extent, in the presence of liver extract or peptone; there was little or no conjugation in either the water or the proteose peptone.

The proteose-peptone cultures used in the foregoing experiments were usually one to two weeks old. Since the "hormone" study indicated that conjugation could take place even in the presence of proteose peptone, providing the medium had supported sufficient previous growth, it was of

interest to determine the influence of age on conjugation of unwashed cultures; 1% proteose-peptone cultures of various ages were diluted tenfold with water, and corresponding cultures of the two strains were mixed in depression plates. A pair of 6-day cultures in 1% yeast extract was similarly treated; 16-hr cultures were diluted only twofold. Results are shown in Table 5.

TABLE 5

Effect of culture medium and age

Hours after mixing	Diluted tenfold			Diluted twofold	
	Proteose peptone 62 day 24 day 6 day			Proteose peptone 16 hr	Yeast extract 16 hr
2	0	0	0	0	0
4	III	0	0		
6	VI	IV	0		
7½	VI	VII	0	0	0
25½	VI	VII	0	0	0
31½	VI	VI	0	0	0
48½	III	III	0	0	0
56½			0	0	0
72		III	0	0	V
79		VII	0	0	III
95		IV		0	IV

Yeast extract cultures consistently showed much earlier conjugation than comparably treated proteose peptone cultures, and it was also shown that addition of yeast extract or similar extracts greatly enhanced conjugation in washed animals grown in proteose peptone. These findings suggested that washing of animals grown in yeast extract might not be necessary or even desirable in obtaining conjugation. Prior to mixing, portions of 10-day-old yeast extract cultures were subjected to one of the following treatments: (a) diluted with distilled water, (b) centrifuged and the animals resuspended in distilled water, or (c) centrifuged, washed once with water, and resuspended in fresh distilled water. As a control, proteose peptone cultures of the same age were diluted and mixed at the same time. The results, as shown in Table 6, demonstrate that the earlier conjugation is obtained without any washing of yeast extract cultures.

The increased reactivity in older cultures, and the failure of fresh proteose peptone to support conjugation in washed animals, are in keeping with the original working hypothesis that depletion of a specific nutrient is a necessary condition for conjugation. The strong reaction of yeast extract

cultures which are still capable of supporting proliferative growth, the adverse effect of washing on the reactivity of animals grown in yeast extract, and the remarkable enhancement of conjugation following the addition of dilute yeast extract to washed animals grown in proteose peptone, all indicate, on the other hand, that an adequate supply of at least some nutrients is essential for conjugation. It appears from this work that optimum conditions for conjugation do not include starvation, but rather a nutrient supply which is qualitatively different from that optimum for vegetative growth.

TABLE 6

Suppression of conjugation by washing of animals
grown in yeast extract.

Hours after mixing	Yeast extract cultures			Proteose peptone
	Diluted	Resuspended	Washed	Diluted
1½	0	0	0	0
2½	III	0	0	0
3½	V	II	0	0
4½	VII	IV	0	0
5½	VII	V	0	0

Reference

1. Ducoff, Howard S. The influence of specific nutrients on the mating reaction in *Tetrahymena pyriformis*. Quarterly Report of Biological and Medical Research Division, Argonne National Laboratory. ANL-5597, p. 15 (1956).

PROGRESS REPORT: THE INFLUENCE OF SPECIFIC NUTRIENTS
ON THE MATING REACTION IN TETRAHYMENA PYRIFORMIS

III. Enhancement of reactivity by certain reducing agents
and by neopyrithiamin

Howard S. Ducoff

The demonstration⁽¹⁾ that the presence of some nutrients favored conjugation between the LF and Ham-3 strains of T. pyriformis has made it possible to screen a great variety of metabolites, antimetabolites, and other compounds for either enhancing or suppressing action. "Enhancing" action is most easily recognized in washed animals from either yeast extract or proteose peptone cultures, or in dilutions of young proteose peptone cultures. The present report is concerned with a few materials which have exhibited rather spectacular enhancing action, and with preliminary experiments designed to elucidate the mechanism of action of these materials.

The first material found to possess enhancing activity was ascorbic acid. It has proven effective in concentrations ranging from 1 to 30 $\mu\text{g}/\text{ml}$, and in both yeast extract and proteose peptone cultures, with or without prior washing. Since the effect of ascorbic acid might be due either to a vitamin function or to its activity as a biological reducing agent, several other reducing agents were tested. Glutathione and cysteine were both very effective, but ferrous iron, in the concentrations employed, was ineffective. As a control, cystine, the oxidized form of cysteine, was also tested; no enhancement was found. Results of one such experiment, using a washed four-day-old yeast extract culture, are shown in Table 7, where the scoring system is the same as that described in the preceding paper.⁽¹⁾

Another material very effective in enhancing conjugation in these strains was the thiamin analogue, neopyrithiamin (NPT), which was active in concentrations ranging from 6 to 60 $\mu\text{g}/\text{ml}$. To test whether the activity of NPT was specifically related to its anti-thiamin effect, NPT was tested on washed yeast extract animals in the presence of added thiamin. The results, shown in Table 8, indicate that neopyrithiamin enhances conjugation by virtue of its antithiamin activity.

It is not at all clear as yet whether or not biological reducing agents and NPT act via similar mechanisms. Experiments are now in progress to examine this question. Neither is it known whether these agents act to "condition" the animals to bring about early reactivity, or whether they affect some later step in a chain of reactions leading to conjugation. This point is probably more amenable to investigation in other forms, assuming that the same agents which enhance conjugation between the LF and Ham-3 strains of T. pyriformis will prove effective in other species.

TABLE 7

Enhancement of conjugation by reducing agents

Hr after mixing	Water	Cysteine 1.3 $\mu\text{g}/\text{ml}$	Cystine 1.3 $\mu\text{g}/\text{ml}$	Ascorbic acid 2 $\mu\text{g}/\text{ml}$	FeCl ₂ 1 $\mu\text{g}/\text{ml}$
2½	0	0	0	0	0
4½	0	IV	0	0	0
16	0	IV	0	IV	0
21	0	III	0	IV	0
23	0	III	0	III	0
40	0	IV	0	IV	0

TABLE 8

Enhancement of conjugation by thiamin antagonism

Hr after mixing	Thiamin 1.25 $\mu\text{g}/\text{ml}$	NPT 15 $\mu\text{g}/\text{ml}$	NPT+ Thiamin
2½	0	III	0
4½	0	III	0
16	0	VI	0
23	0	VII	0
40	II	V	0
48	0	III	0

Reference

1. Ducoff, Howard S. 1956. The influence of specific nutrients on the mating reaction in Tetrahymena pyriformis. II. Experiments with complex media. This report, p. 25.

ON A METHOD OF DETERMINING ASSOCIATION BETWEEN UNORDERED PAIRS

S. A. Tyler and E. L. Powers

A frequently occurring problem in biological investigations is the determination of the degree of association between members of a family. When ordinal values can be assigned to the members, standard statistical methods can be employed. In some instances, however, an ordering of events is either impossible or undesirable. For example, it may be desirable to recognize (a) the extent of correlation between the survival ratios of the progeny of sisters after treatment, or (b) the relationship between litter-mates on the basis of some quantized observation or measurement. In both of these cases an ordering of the events, regardless of the criterion used, would vitiate the test result. A statistic commonly used to indicate the degree of relationship between family members when an ordering cannot be accomplished is the intraclass correlation coefficient, r' .⁽¹⁾ The sampling distribution of r' , when the values are normally distributed and the number in each family is equal, can be approximated, thus giving some criterion upon which a judgment of the significance of r' can be made.^(2,3) However, at times, an investigator is unwilling to assume the normal hypothesis and thus is unable to utilize the intraclass correlation coefficient for assessing the degree of similarity between family members. For this reason, we consider below, in the simple case of a set of n families each containing two members, a non-parametric method of determining the extent of correlation within pairs that is independent of the distributional form of the values as well as of the differences in the actual quantitative values of the observed pairs.

Let Y_{ij} equal the defining measure of the j th member of the i th family ($i = 1, 2, \dots, n$; $j = 1, 2$). Since the relative closeness of members of a pair and not the actual magnitude of Y_{ij} is important, we may rank according to magnitude the $2n$ sample values,*

$$Y_{ij} \xrightarrow{\quad} X_{ij}$$

where X_{ij} is a positive integer $\leq 2n$ and is the rank of the corresponding Y_{ij} value. It is evident that the greater the association (or correlation) in the positive sense between pairs, the smaller will be the difference between the ranks of pairs and the smaller will be the sum of the rank differences. In fact, if $S = \sum_i |X_{i1} - X_{i2}| = n$, then members are perfectly correlated in a

*To evade, at this time, the problem of ties in ranks, we assume that the $2n$ values are distinct.

positive sense. Inversely, a negative correlation among members implies a large sum S . Thus, we may test a set of families for correlation among member pairs by comparing the distribution of observed sums S with that distribution expected from random sets of ranks.

Consider a random set of n pairs consisting of ranks from 1 to $2n$: $X_{11}, X_{12}; X_{21}, X_{22}; X_{31}, X_{32}; \dots; X_{n1}, X_{n2}$. The total number of distinct ways⁽⁴⁾ of choosing these n pairs is equal to $(2n)! / 2^n \cdot n! = \prod_{i=1}^n (2n - 2i + 1)$. We have observed that the sum of the absolute differences $S = \sum_i |X_{i1} - X_{i2}|$ has a minimum of n and a maximum of n^2 and is defined only for the $r = \frac{n^2 - n + 2}{2}$ discrete values $S_p = n + 2(p-1)$, $p = 1, 2, \dots, r$. By enumeration, the frequency distributions of sums for 1 to 5 pairs were found and are tabulated in Table 9.

TABLE 9

Frequency distributions of the sums of the absolute differences
between sets of 1 to 5 paired ranks

Sums	1 pair		2 pairs		3 pairs		4 pairs		5 pairs	
	Count	%	Count	%	Count	%	Count	%	Count	%
1	1	100.0								
2			1	33.3						
3					1	6.6				
4			2	66.7			1	0.95		
5					4	26.7			1	0.10
6							6	5.71		
7					4	26.7			8	0.85
8							12	11.43		
9					6	40.0			24	2.54
10							20	19.05		
11									50	5.29
12							24	22.86		
13									88	9.31
14							18	17.14		
15									108	11.43
16							24	22.86		
17									156	16.51
19									150	15.87
21									144	15.24
23									96	10.16
25									120	12.70
Total	1	100.0	3	100.0	15	100.0	105	100.00	945	100.00

To approximate the distribution of sums for 6 or more pairs, random samples from appropriate populations will be drawn using high speed computing equipment. Against these distributions, sums from sets of observed pairs can be tested for compatability using "Chi-square." The following biological problem serves as an example of an application of this method.

When a single cell divides mitotically, it is generally assumed that the two cells produced are genically identical. If this is true, a test on these two cells, or sublines derived from them, should give similar results if the test response is genetically determined. Conversely if the responses are similar the inference may be allowed that they are regulated by determiners distributed equally to the two daughter cells. In Paramecium aurelia a response frequently used in radiobiological experiments is survival (or death) after an intracellular nuclear reorganization called autogamy. There is cytological(5) and genetic(6) evidence that the cells emerging are homozygous. Since the particular chromosomes surviving reduction are randomly chosen, a certain fraction of the cells of a clone should survive if the original cell (from which the clone is derived) is heterozygous for recessive lethal genes in its micronuclei. If the amount of death observed is due primarily to the micronuclear lethal genes, then two clones derived from sister cells should evidence degrees of survival after autogamy much closer than any other two clones from randomly chosen treated cells (i.e., not from sister cells).

Our test was as follows. From a number of clones derived from X-rayed paramecia, isolations of single cells were made. After one division each of the single cells was isolated, and these sister cells were allowed to produce cultures. In six such cultures (3 pairs of sublines) autogamy was induced and the fraction of the emerging cells unable to produce vigorous clones was noted. One particular result is presented in Table 10.

TABLE 10

Post autogamous deaths among emerging cells

Line	Subline	% postautogamous death	Rank	Absolute differences between ranks
1	a	91.6	3	1
	b	94.4	2	
2	a	80.6	6	2
	b	87.8	4	
3	a	100.0	1	4
	b	83.3	5	
				Sum 7

The table demonstrates the method of analysis. In this instance the sum of the absolute differences between ordinal values within a line is 7. As noted above the minimum number possible is 3 and the maximum is 9. Since it is necessary to measure the distribution of these sums to compare it with the distribution expected on a random basis, 17 such analyses were made with the result shown in Table 11.

TABLE 11

Distribution of sums of differences between
ordinal ranks in pairs of numbers

Sum	Expected random		Observed	
	frequency	%	frequency	%
3	1	6.66	3	17.65
5	4	26.67	5	29.41
7	4	26.67	3	17.65
9	6	40.00	6	35.29

The Chi-square analysis of the result gave $X_0^2 = \frac{\sum (E-C)^2}{C} = 21.919$; for 4 degrees of freedom. The probability of $X^2 \geq X_0^2$ is less than 0.001. Therefore, it seems reasonable to conclude that the distribution of the observed values is not random. Since there seems to be an accumulation of the lower values, positive correlation between pairs is indicated - that is, each subline is more like its sister subline than it is like the other sublines in the group.

The method is being applied currently to larger series and, when theoretical distributions become available, to higher numbers of pairs of values.

References

1. Fisher, R. A. Statistical Methods for Research Workers, 12th edition, revised. Oliver and Boyd, London (1954).
2. Yule, G. V., and M. G. Kendall. An Introduction to the Theory of Statistics, 14th edition. Griffin and Co., Ltd., London (1950).
3. Kendall, M. G. The Advanced Theory of Statistics, Vol. 1, 3rd edition. Griffin and Co., Ltd., London (1947).
4. Feller, W. An Introduction to Probability Theory and Its Applications, Vol. 1. John Wiley and Sons, Inc., New York (1950).
5. Diller, W. F. Nuclear reorganization processes in Paramecium aurelia, with descriptions of autogamy and "hemixis." J. Morphol. 59:11-52 (1936).
6. Sonneborn, T. M. Recent advances in the genetics of Paramecium and Euplotes. Adv. in Genetics 1:263-357 (1947).

THE MEAN DELAY IN THE APPEARANCE OF THE PHENOTYPE FOR PHAGE RESISTANCE IN ESCHERICHIA COLI

Herbert E. Kubitschek and Harold E. Bendigkeit

Mutants resistant to the bacteriophage T5 are known to be induced by exposure of E. coli strain B/1,t to caffeine during growth. We have measured the mean delay for the appearance of this phenotype for E. coli in the chemostat with growth limited by tryptophan.

Our method has appreciable advantages over the previous plate culture techniques in which bacteria were plated from a culture in lag phase onto an agar surface, allowed to grow, and sprayed with phage:

1. Bacteria from the receiving tube of a chemostat are dispersed in liquid and therefore are all susceptible to infection by the phage, whereas bacteria inside microcolonies on an agar surface are shielded for some time from the phage sprayed upon the colonies.

2. In the chemostat, the parental culture grows uniformly following the application of a mutagen. However, when the mutagen is applied to bacteria in lag phase, which is conventional in the plate culture method, a loss in resolution occurs because bacteria emerge irregularly to the growth state.

3. With the chemostat, no correction need be made for mutants occurring spontaneously upon the nutrient agar plates used for titering them.

We have measured the delay in the appearance of phage resistance to be about 3.4 generations, and it appears to be independent of growth rate for generation times longer than 3 hr (Fig. 16). At growth rates higher than this the culture is in continuous growth but it is probably no longer limited by tryptophan, as is indicated by a decrease in bacterial titer.

The delay appears also to be constant and independent of the following parameters: bacterial titer, caffeine concentration (Fig. 17), temperature, and culture volume. The same delay occurs for both T5 resistant variants, the E and L strains.⁽¹⁾ The delay is also constant for bacteria exposed to concentrations of 2×10^9 phage per ml or greater. The mean delay under different conditions is given in Table 12. The standard error is estimated with the assumption that every determination has the same validity.

Figure 16. The independence of the mean delay upon generation time. The dotted lines indicate the standard errors. The average of all mean delays for generation times greater than 3 hr is 3.42 generations, and is indicated by the heavy horizontal line.

Figure 17. Independence of the mean delay upon caffeine concentration. The generation times are indicated, with the number of measurements at each time given in parentheses if more than one measurement was made. The solid horizontal line indicates the average of all measurements on the graph and the dotted lines show the extent of the standard error.

TABLE 12

Measurements of mean delay

Experimental parameter varied	Range of generation time, hr	Number of measurements	Mean delay, generations	Standard error, generations
Standard conditions: all measurements at 37°, 450 mg/l caffeine, bacterial titer $\sim 2 \times 10^8$ /ml	3 - 16.6	72	3.42	.10
Bacterial titer: 10-fold decrease	3 - 16.6	12	3.4	.3
Temperature: 26°	7.7 , 16.6	8	3.6	.3
Strain and culture conditions:				
E strain on nutrient agar	3.2 - 13.2	52	3.48	.12
L strain on nutrient agar	4 - 13.2	7	3.5	.2
E strain, nutrient agar supplemented with Fe	5.6 - 11.3	4	3.5	.03
L strain, nutrient agar supplemented with Fe	5.6 - 11.3	4	3.2	.6

Reference

1. Kubitschek, H. E., and H. E. Bendigkeit. A new variant for T5 resistance in Escherichia coli. Quarterly Report of Biological and Medical Research Division, Argonne National Laboratory. ANL-5576, p. 21 (1956).

TOXICITY OF LOCALLY DEPOSITED YTTRIUM⁹¹ IN MICE.

I. EXPERIMENTAL DESIGN AND GROSS PATHOLOGY

Miriam P. Finkel, Hermann Lisco,
Katherine A. Lathrop, and Austin M. Brues

The local effects of an insoluble beta emitter deposited within the body was studied by injecting mice either under the skin of the back or within the muscles of the right hind leg with a solution containing an insoluble suspension of $Y^{91}PO_4$. This isotope decays with a half-life of 57.5 days to stable zirconium; its beta particle has a maximum energy of 1.55 Mev.

The mice were young adult hybrids (designated ABC) supplied by the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine. The experimental design is given in Table 13. A few of the mice were sacrificed at specified times in order to provide information on the early histopathological changes at the injection site as well as to furnish data on local retention and widespread distribution of the material. The remainder were permitted to live until natural death occurred, or they were killed when moribund. Gross autopsies were performed unless autolysis was advanced; tissues were taken for microscopic study in many instances. A preliminary note on the induction of local tumors has already appeared.⁽¹⁾

TABLE 13

Experimental design

Total Dose, mc	Injection site	Total number of animals	Number of autopsies	Sex
1.5	intramuscular	2	2	M
0.5	intramuscular	2	2	F
0.3	intramuscular	6	3	M
0.1	intramuscular	12	11	M
0.03	intramuscular	20	13	M
0.01	intramuscular	20	19	F
1.5	subcutaneous	2	2	F
0.5	subcutaneous	2	2	F
0.3	subcutaneous	6	6	F
0.1	subcutaneous	12	9	F
0.03	subcutaneous	20	12	F
0.01	subcutaneous	20	18	M
0	control	20	20	M F

Survival. The experimental and control animals were about equally divided between males and females. Since the survival curves for each sex among the control animals were very similar, they have been combined in Figures 18 and 19. Except for a few instances of urogenital infection resulting from fighting among the males, there was no evidence that any of the observations were dependent upon sex. Approximately 250 days after injection a lung infection became apparent throughout the colony. The disease reached epidemic proportions only in the cage that housed the mice that had received 0.01 mc of Y^{91} subcutaneously. Over 60% of the control animals lived for 500 days after the beginning of the experiment. However, all but one of these succumbed before 550 days to an acute intestinal infection. The 500-day survivors among the experimental groups were similarly affected.

In Figure 18 it can be seen that the intramuscular injection of 0.01 and 0.03 mc resulted in a moderate decrease in survival time; the median survival time was about 390 days. Doses of from 0.1 to 1.5 mc led to 50% mortality at approximately 260 days. Within this latter range increased dose did not decrease survival time. The survival curves after subcutaneous injection (Figure 19) are not as clear-cut, even when the pneumonia deaths among the animals of the 0.01 mc group are excluded. Except for the early demise of 3 of the 4 mice that received 0.5 or 1.5 mc, the pattern is quite haphazard. This is probably due to individual differences in loss of the injected material. Unlike the intramuscular injections, which placed the Y^{91} deep within the muscles of the leg, the subcutaneous injections deposited it under the skin of the back, where a small variation in depth could lead to a great variation in extent of superficial necrosis and subsequent sloughing of injected material.

Observations at autopsy, excluding injection-site lesions. In addition to the lung and intestinal infections mentioned above, liver and kidney diseases were common. The livers were frequently large, fleshy, and mottled. The kidneys were often small, pale, and scarred, or they were abscessed or polycystic. There was no indication that these conditions were associated with dosage (Table 14).

A localized or generalized dermatitis involving epilation, scaling, oozing, ulceration, and crusting was not uncommon. Included in Table 14 are only those lesions that were not associated with the site of injection.

Lung tumors were frequently seen at autopsy; gross appearance indicated that most, if not all, were benign. The distribution of these tumors among the dosage groups, as well as the distribution of the other neoplasms listed in Table 14, does not suggest an association between tumor occurrence and irradiation. The only suspicious data are the 4 lymphoid tumors among those animals that received 0.01 mc intramuscularly. However, this is not an unusually high incidence; rather, experience with ABC mice indicates that the incidence among the control animals was unusually low.

Figure 18. Survival after intramuscular injection of $Y^{91}PO_4$.

Figure 19. Survival after subcutaneous injection of $Y^{91}PO_4$.

42

TABLE 14

Summary of autopsy findings (excluding injection-site lesions)

Total dose, mc	Liver disease	Kidney disease	Lung disease	Intes. infec.	Dermatitis	Lung tumors	Other tumors
Intramuscular injection							
1.5-0.5	25	50	25	0	0	0	25 (1 liver)
0.3	100	100	0	0	0	33	0
0.1	36	64	18	0	9	18	0
0.03	38	38	8	0	15	54	15 (1 liver; 1 lymphoid)
0.01	37	32	11	5	0	32	32 (1 liver; 4 lymphoid; 1 uterine)
Subcutaneous injection							
1.5-0.5	0	0	25	25	0	25	0
0.3	50	100	0	17	50	30	0
0.1	44	78	33	0	0	0	0
0.03	8	42	25	25	17	58	8 (1 lymphoid)
0.01	28	44	61	22	17	6	6 (1 benign mesodermal)
Control							
0	25	60	15	50	20	45	10 (1 papilloma; 1 skin carcinoma)

Values represent per cent of autopsies at which the specified lesions were found.

Injection-site lesions after intramuscular injection. Complete, spontaneous amputation of the injected leg occurred in the 4 animals that received 0.5 or 1.5 mc of $Y^{91}PO_4$ intramuscularly (Table 15). Two of the 3 that received 0.3 mc suffered amputation of the entire leg, and a fibrosarcoma developed in the stump of one. The third animal of this group was autopsied 261 days after injection, at which time the site was marked

only by an 8 mm area of ulceration and keratosis. Sections of this region are not available for microscopic study; however, some lesions with similar gross appearance have proved to be epidermoid carcinomas.

TABLE 15

Summary of local lesions after
intramuscular injection

Total dose, mc	Greying	Epilation	Ulceration	Amputation	Carcinoma of skin	Hemangioendothelioma	Fibrosarcoma or Anaplastic sarcoma	Osteogenic sarcoma	$\frac{\text{Number of tumors}}{\text{Number of animals}} \times 100$
1.5-0.5	100	0	0	100	0	0	0	0	0
0.3	100	0	67	67	0	0	33	0	33
0.1	55	55	36	27	27	0	18	0	45
0.03	62	38	23	46	8	38	15	23	92
0.01	58	5	5	0	5	26	11	26	68

Values, except in last column, represent per cent of autopsies at which specified lesions were found.

Among the 11 animals in the 0.1 mc group, one lost the entire injected leg and two suffered amputation at the knee. The others displayed various degrees of local necrosis and calcification, and three developed epidermoid carcinomas. Two of these three had local sarcomas as well; one was undifferentiated and the other was a fibrosarcoma. Thus there was a total of 5 tumors among the 11 animals that received 0.1 mc of Y⁹¹.

Spontaneous amputation among the animals of the 0.03 mc group involved the foot alone in the majority of cases. Two of the 13 animals had 2 local tumors apiece, and 8 had single tumors. At 0.01 mc there were no cases of amputation, but 14 of the 19 animals displayed tumors at the injection site.

Of the 31 local tumors observed among the mice that received intramuscular injections of $Y^{91}PO_4$, 10 were hemangioendotheliomas, 8 were osteogenic sarcomas, 6 were fibrosarcomas, 5 were carcinomas of the skin, and 2 were undifferentiated sarcomas. The only comparable tumor at any other location among the animals of this experiment was an epidermoid carcinoma that appeared on the back of the longest survivor, a control female mouse that lived 794 days after the beginning of the experiment.

The distribution of injection-site tumors among the animals that received intramuscular injections was found to agree reasonably well with a Poisson distribution (Table 16). The differences between the observed and the theoretical frequencies probably reflect inadequacies of the data rather than deviations from randomness (e.g., poor roentgenograms and deficiencies of histological specimens).

TABLE 16

Distribution of local tumors

No. of tumors per animal	Number of animals	
	Observed	Calculated (Poisson)
0	19	22.8
1	23	16.0
2	4	5.6
3	0	1.3

Injection-site lesions after subcutaneous injection. After the acute phase of injury had been passed, the incidence and severity of injection-site lesions in the skin displayed little if any relation to dose and to time (Table 17). This was probably the result of differential sloughing of the injured skin: the larger the dose, the larger the area of necrosis, and therefore, the greater the loss of the injected material. However, the lesions that appeared in the deeper tissues underlying the injection site, such as skeletal muscle, spine, and kidney, did vary with the dose. At 0.01 mc only the 3 longest survivors showed radiation-induced kidney or muscle changes, whereas among the 0.03 to 0.3 mc groups similar lesions were seen in a larger proportion of the animals and at all time intervals. The early loss of most of the injected material probably accounts for deeply situated lesions being seen in only one of the four animals that received 0.5 or 1.5 mc.

TABLE 17

Summary of local lesions after
subcutaneous injection

Total dose, mc	Greying	Epilations	Ulceration	Necrosis of muscle, spine, or kidney	Carcinoma of skin	Fibrosarcoma	Osteogenic sarcoma	Number of tumors Number of animals x 100
1.5-0.5	25	50	0	25	0	0	0	0
0.3	67	50	33	67	0	0	0	0
0.1	33	44	33	89	11	0	0	11
0.03	92	50	33	42	8	8	17	33
0.01	72	44	33	17	17	0	0	17

Values, except in last column, represent
per cent of autopsies at which specified
lesions were found.

Eight local tumors appeared in 7 of the 41 animals that were autopsied 200 or more days after subcutaneous injection. Five were epidermoid carcinomas, 2 were osteogenic sarcomas of the lumbar spine, and one was a fibrosarcoma.

Summary

One hundred twenty-four hybrid Bar Harbor mice (ABC) received intramuscular or subcutaneous injections of $Y^{91}PO_4$. Twenty additional animals served as controls. Approximately 250 days after injection a lung infection was apparent throughout the experiment, but only one cage was severely affected. Approximately 500 days after injection an acute intestinal infection proceeded to eliminate most of the survivors.

In general, survival time decreased with increasing intramuscular dose. The survival data after subcutaneous injection was quite erratic, probably because of individual differences in loss of injected material.

Injection-site lesions included greying, epilation, ulceration, and tumor formation. Intramuscular injection was frequently followed by amputation of all or a part of the injected leg; subcutaneous injection often led to severe irradiation injury of the underlying muscle, spine, and kidney.

Thirty-nine local tumors were observed, 31 after intramuscular injection and 8 after subcutaneous injection. There were 10 epidermoid carcinomas, 10 hemangioendotheliomas, 10 osteogenic sarcomas, 7 fibrosarcomas, and 2 undifferentiated sarcomas.

Reference

1. Lisco, H., M. P. Finkel, and A. M. Brues. Local tumors following injection of $Y^{91}PO_4$. Quarterly Report of Biological and Medical Research Division, Argonne National Laboratory. ANL-5518, p. 115 (1956).

PROGRESS REPORT: RADIATION EFFECTS ON THE
PARAMECIUM BURSARIA MATING REACTION.

Light, Ultraviolet and Post-Ultraviolet
Light Effects on Conjugants

Charles F. Ehret, Antoinette Votava and Lora Harrer*

The use of separation time (or duration of mating) of conjugating paramecia as a measure of cell damage was discussed recently.⁽¹⁾ At that time it was pointed out that both agglutination and holdfast formation function as selective processes which may contribute to the character of those dose-effect curves in which delay in separation-time (a terminal step in the mating reaction sequence) was the measure of effect.

One method of eliminating the selective action of early mating reaction events is to irradiate the animals after the early events have occurred, i.e., during conjugation. This approach was taken in a series of experiments consisting of two phases. First, the separation times resulting when populations of each mating type were irradiated before mating were compared with those resulting when populations of conjugants were irradiated after mating. Secondly, the effects of light, ultraviolet (UV), and UV + light were measured as a function of the time after mating at which these treatments were applied (5.5, 8.1, 10.2 and 12.0 hr). Exposure methods and mating types were as previously described; the stocks were FM 2-3 chl - mt B and Wu 5 chl + mt D, and the temperature was 24.5°C.

The effects of UV, and the subsequent photoreactivation, are shown in Figure 20, in which results obtained when populations of single animals of both mating types were irradiated at 0920,** placed in darkness or illuminated for 1 hr, and then mixed at 1030 (in the early reactive period), are compared with results obtained when animals mixed at 1030 were irradiated at 1415 (3.75 hr after mating) as conjugants, and then placed in darkness or in light for 1 hr. At all other times after 0920, animals remained in darkness until reading time 23 hr later. Under these conditions, conjugants appear to be slightly more resistant rather than more sensitive to UV than single animals -- although selection in the direction opposite to that previously discussed may be involved (e.g., the most damaged cells when the reference point is agglutination or holdfast formation may be screened from some of the damage done to the conjugation-timing mechanisms). However,

*Student Aid from Marycrest College.

**The actual times are given because of the diurnally rhythmical character of the mating reaction.

Figure 20.

The relationship between time required for 40% of the conjugants to separate and UV dose. The single animals were irradiated first, and then were mated 1 hr later. The conjugants were irradiated 3.75 hr after mating. "Reactivating" light was applied in each case immediately after the UV dose.

the gross character of the curves is similar for both UV and UV + light, and compatible with the curves previously obtained when animals of only one mating type had been irradiated (Fig. 2-3 in reference 1).

The plan of the second phase is shown in Table 18. Animals were mated at 1035 in the early reactive period; the mating reaction and conjugation took place in 500 lux white light; and conjugants were isolated from the reaction mixture at 1500-1600. The indicated treatments were then applied; the UV dose was 120 seconds at $5 \mu\text{W}/\text{mm}^2$, and each light exposure was for 1 hr, with the exception of No. 17, in which the light exposure was continuous. Category 18 was examined cytologically with the phase contrast microscope at intervals throughout the experiment. The results are given in Figures 21-23. From Figure 21 it is clear that until early prophase, light has little or no effect on the separation time of these animals which have been in light throughout the early reactive period. However, 1 hr exposure to light of a population between crescent prophase and 1st meiotic division caused a significant short delay; and continuous exposure (No. 17) caused a long delay in 10% of the animals. Figure 22 again shows that the photoreactivation of separation time delay is not a selection artifact (compare No. 3 and 4); furthermore, the magnitude of photoreactivation was not diminished when reactivating light was applied within 2.5 hr after UV, i.e., up to the time of early prophase (No. 5). Photorecovery was decidedly less when light was applied 4.6 hr after ultraviolet treatment, during crescent to late prophase (No. 6). However, instead of diminishing further, the magnitude of photorecovery again increased after prophase, when the population was in metaphase-telophase, 6.5 hr after UV (No. 7).

TABLE 18

Schedule of treatments applied to conjugating paramecia mated at 1035.
The category code numbers identify curves in Figures 21-23.

Category No.	Time of treatment			
	1605 (5.5)*	1840 (8.1)*	2045 (10.2)*	2235 (12.0)*
1	Dark (dk) —————→			
2	Light (Lt) 1 hr;dk —————→			
3	UV + dk —————→			
4	UV + Lt 1 hr;dk —————→			
5	UV + dk —————→	+ Lt 1 hr;dk —————→		
6	UV + dk —————→		+ Lt 1 hr;dk —————→	
7	UV + dk —————→			+ Lt 1 hr;dk
8	dk —————→	UV + dk —————→		
9	dk —————→	UV + Lt 1 hr;dk —————→		
10	dk —————→	Lt 1 hr;dk —————→		
11	dk —————→		UV + dk —————→	
12	dk —————→		UV + Lt 1 hr;dk —————→	
13	dk —————→		Lt 1 hr;dk —————→	
14	dk —————→			UV + dk
15	dk —————→			UV + Lt 1 hr;dk
16	dk —————→			Lt 1 hr;dk
17	Lt —————→			
18	Cytological control	Early prophase	Crescent prophase to late prophase	Late prophase, metaphase, anaphase, and complete telophase

*Figures in parentheses represent hours after mating.

Figure 21.

Figure 22.

Figure 23.

The relationship between exconjugant separation and time after initial mating. The identifying code numbers are explained in Table 18.

Sensitivity to ultraviolet appeared to increase slightly until late prophase (Fig. 23, Nos. 8 and 11; compare with Fig. 22, No. 3), and then to decrease (No. 14). The most photoreversible stage (in terms of UV + immediate-light-application time and the corresponding "dose reduction" as applied to curves of Figure 20) is pre-prophase (Nos. 3,4), followed in order by crescent to late prophase, (Nos. 11,12) and lastly by late prophase to telophase (Nos. 14,15) and early prophase (Nos. 8,9). However, if corresponding control corrections are applied (Fig. 21), then the order becomes 3,4; 14,15; 11,12; 8,9. Therefore photoreactivation appears consistently least effective between early and late prophase.

Reference

1. Ehret, C. F. and J. E. Fraembs. Holdfast formation and separation time as measures of UV-induced cell damage. Quarterly Report of Biological and Medical Research Division, Argonne National Laboratory. ANL-5518, pp. 101-103 (1956).

PURINE RIBOTIDE-AMINO ACID INTERACTIONS I.

SPECIFICITY OF COMPONENTS OF ADENOSYLMETHIONINE-FORMING REACTION IN RAT LIVER EXTRACTS

Peter D. Klein

The formation of adenosylmethionine (AM) from methionine and adenosine triphosphate (ATP) according to the reaction

has been previously described.^(1,2,3) To date, no intimate knowledge of the sequence of reactions leading to the formation of this compound has been gained. It has been proposed by Cantoni⁽⁴⁾ that in the dismutation of the adenosine triphosphate, the first step is the liberation of P_i , followed by cleavage of pyrophosphate from the remaining molecule. Little or no work has been published concerning the specificity of the components of this reaction with respect to the alpha amino carboxyl end of methionine, the number of phosphates necessary, or the constituent base of the phosphate donor.

This report deals with studies of methionine, its derivatives and analogs, the mono-, di-, and tri-phosphates of adenosine and the riboside triphosphates adenosine, cytosine, guanosine and uridine as participants in the reaction described.

Method

The enzyme source was a rat liver extract prepared by homogenizing one part liver in four parts 0.1 M sodium acetate followed by centrifugation at 100,000 x g. Further purification has been carried out; however the product does not appear to differ in requirements from the original extract.

Measurement of adenosylmethionine formation. Following incubation of the enzyme together with the appropriate buffer, activators and other constituents, the reaction was terminated by the addition of an equal volume of cold 2 N perchloric acid. Inorganic phosphate analyses were performed on the supernatant using either the method of Lowry and Lopez⁽⁵⁾ or that of Fiske and SubbaRow.⁽⁶⁾ The two yielded identical results. The formation of AM was calculated from the difference in P_i between tubes with and without added methionine. The micromoles of inorganic phosphate divided by three are equivalent to micromoles of AM formed. Alternatively, the amount of AM formed was measured spectrophotometrically by a method to be described.

Results and Discussion

When various configurations of methionine, or ethionine or the hydroxy analog were tested, L-methionine displayed the highest activity (Table 19). The racemic mixture appeared slightly less effective on the basis of phosphate determination, while the D configuration had only one-third as much activity as the L form. Both ethionine and alpha-hydroxy, gamma-methylmercaptobutyric acid were totally inactive.

TABLE 19

Specificity of amino acid constituent

Methionine or analogue	AM formed	
	$P_i/3, \mu M$	260 m μ , μM
L-methionine	1.59	1.45
DL-methionine	1.34	1.54
D-methionine	0.55	0.44
L-ethionine	0.20	0.00
α -hydroxy γ -methyl- mercaptobutyric acid	0.12	0.00

Conditions: 100 μM $MgCl_2$; 50 μM Tris buffer, pH 7.4; 5 μM ATP neutralized to pH 7.4; 8 μM glutathione neutralized to pH 7.4; 10 μM methionine or analog, 0.5 ml enzyme extract; final volume 1.00 ml; incubated for 1 hr at 37°C.

From results using adenosine monophosphate (AMP), adenosine diphosphate (ADP), and ATP (Table 20), it was also evident that the triphosphate form was preferentially used to form adenosylmethionine. Some activity was discernible when ADP was used. This was undoubtedly the result of myokinase action which converts two molecules of ADP into one each of AMP and ATP. Even in the event that the activity involved ADP directly, it can be seen that the reaction formed only 40% as much adenosylmethionine from the ADP as from the same initial concentration of the triphosphate. No difference in phosphate liberation could be measured in the reaction utilizing AMP, since the control tube, containing no methionine, completely dephosphorylated the AMP. The small amounts of adenosylmethionine found by the spectrophotometric method may have resulted from phosphorylation of the AMP to higher phosphate esters by residual glycolytic action in the extract.

TABLE 20

Specificity of triphosphate requirement

Phosphate source	AM formed		
	n	P _i /n, μ M	260 m μ , μ M
ATP	3	1.58	1.22
ADP	2	0.64	0.59
AMP	1	0*	0.23

Conditions: As in Table 19. Adenylic compounds present in 5 μ M concentration.

*Control tube without added methionine completely dephosphorylated all of AMP present within period of incubation.

The specificity of nucleotide purines is examined in Table 21. Again, the ATP was the only nucleotide which promoted synthesis of the purineriboside-methionine. Small amounts of adenosine compounds present in the extract permitted a small but measureable amount of adenosylmethionine formation. Such formation is attributable to the transphosphorylation from the other purineriboside triphosphates to the adenosine series. When this source is exhausted, synthesis of adenosylmethionine ceases. This is despite a greatly increased dephosphorylation of the cytosine, guanosine and uridine triphosphates.

TABLE 21

Specificity of nucleotide base

Base*	AM formed	
	P _i /3, μ M	Corrected,** μ M
ATP	1.86	1.86
CTP	0.15	0
GTP	0.28	0.08
UTP	0.07	0

Conditions as in Table 19.

*A = adenosine, C = cytosine, G = guanosine, U = uridine.

**Corrected for 0.21 μ M adenosine compounds (260 m μ) present in extract available for transphosphorylation.

The specificity for the reaction sequence leading to the formation of adenosylmethionine may then be described as follows: The amino acid must have the natural L configuration, the alpha position must contain the amino group, and the substituent of the mercapto group must be a methyl group. The reaction requires an intact ATP molecule, i.e., the terminal phosphate of ATP is essential and the purine constituent must be adenine.

It is evident from these considerations that the reaction concerned is one involving a high degree of specificity. While not unexpected, this is at variance with similar reactions in yeast, where, so far, ethionine, the hydroxy analogue and the D configuration are acceptable to the organism.⁽⁷⁾ However, the question of modifications of the molecules before their incorporation into adenosylmethionine has not yet been answered.

References

1. Cantoni, G. L. The activation of methionine. *J. Biol. Chem.* 189: 745-753 (1951).
2. Schlenk, F., and R. E. DePalma. Progress Report: The metabolic relation between adenine and sulfur amino acids. Isolation of S-adenosylmethionine from yeast. Quarterly Report, Biological and Medical Research Division, Argonne National Laboratory, ANL-5518, pp. 134-139 (1956).
3. Klein, P. D. The activation of methionine for transmethylation. Quarterly Report of Biological and Medical Research Division, Argonne National Laboratory, ANL-5426, pp. 69-73 (1955).
4. Cantoni, G. L., and J. Durell. Methionine activating enzyme. *Fed. Proc.* 15: 229 (1956).
5. Lowry, O. H., and J. A. Lopez. The determination of inorganic phosphorus in the presence of labile phosphate esters. *J. Biol. Chem.* 162: 421-428 (1946).
6. Fiske, C., and Y. SubbaRow. The colorimetric determination of phosphorus. *J. Biol. Chem.* 66: 375-400 (1925).
7. Schlenk, F. Personal communication.

PROGRESS REPORT: RADIATION STUDIES IN BIRDS

Early Changes in Plasma Glucose Concentration Following
X-Irradiation in Adult Chickens

S. Phyllis Stearner, Margaret H. Sanderson, and Emily J. Christian

It has been generally assumed that the liver of higher vertebrates is a relatively radioresistant organ because histopathological changes have not been demonstrated following exposure to ionizing radiations. Alterations in fat and glycogen content, however, have been reported, and enzyme studies indicate that metabolic changes may occur.⁽¹⁾ Within a few hours after irradiation, changes in the livers of chicks have been noted, e. g. hemorrhages, jaundice and decrease in fat and glycogen content.^(2,3) Functional change is also indicated by an early decrease in liver circulation,⁽⁴⁾ a decrease in plasma protein and an increase in plasma amino acid content.⁽³⁾ The latter is apparently a generalized response, for it could not be correlated with severity of the early reaction in individual chicks. Plasma glucose concentration, on the other hand, showed little change, but in a few very sick chicks a significant increase was observed.⁽³⁾

In adult chickens, focal necrosis occurs in the liver within a few hours after X-irradiation,⁽⁵⁾ and there is also early evidence of decrease in hepatic circulation.⁽⁶⁾ The effect of total body X-irradiation on the plasma glucose of adult roosters is reported here.

Glucose concentration of plasma was determined according to the method of Folin. This procedure also measures nonglucose reducing substances, but, since they are concentrated in the red cells, they should influence the plasma values only slightly. Roosters aged 6-9 months were fasted about 16 hr before a control blood sample was taken, and were again fasted before exposure to 1200 r total-body X-ray at 43 r/min. After irradiation, blood samples were taken at 1, 3 and 6 hr, or terminally if death occurred earlier. Hematocrit determinations were made to indicate changes in plasma volume.

Results are shown in Table 22. Mean values for glucose concentration were increased 75% at 6 hr after exposure and terminal values were increased 110%. While the plasma volume is shrinking, as indicated by the increased hematocrit values, nevertheless, as can be calculated from data in the table, the total circulating glucose is substantially increased.

We do not know whether the observed hyperglycemia results from increased glycogenolysis or from decreased glycogenesis in the liver, or whether it results indirectly from other physiological processes. However, alterations in liver function have been described in conditions of shock, and the hyperglycemia found in such cases has been reported to result from an increased glycogenolysis.

TABLE 22

Change in plasma glucose (mg %) and hematocrit (HCT) values in
6-8 month roosters after 1200 r X-irradiation at 43 r/min.

Animal	0-1 day (control)		1 hr	3 hr		6 hr (or terminal)		Survival time, hr
	HCT	glucose	- glucose	HCT	glucose	HCT	glucose	
1	41	238	317	53	364	63	540*	4
2	42	240	340	51	280	60	395*	6-1/2
3	37	249	271	43	299	57	512*	6-1/4
4	43	260	279	50	263	60	466*	6
5	-	236	-	-	286	-	547*	5-1/2
6	-	237	-	-	250	-	534*	5-1/2
7	-	199	221	-	280	-	380	7
8	49	259	285	58	337	62	310	7-1/2
9	-	240	246	-	243	-	399	8-16
10	46	251	275	55	328	57	431	12-16
11	41	238	331	49	296	-	524	12-14
12	48	247	294	56	305	56	419	22-24
13	43	236	280	50	323	53	448	23-1/2
14	37	216	328	46	323	52	421	7 days
15	49	244	283	57	368	60	396	8 days
16	46	222	314	52	349	51	467	9 days
Mean	43	238	290	52	306	56	419 (6 hr)	
						60	501 (terminal)	

* = terminal

References

1. Thomson, J. F. Vertebrate radiobiology (lethal actions and associated effects). *Ann. Rev. Nuclear Science*, 4:377-400 (1954).
2. Stearner, S. P. The effect of variation in dosage rate of roentgen rays on survival in young birds. *Am. J. Roentgenol.* 65:265-271 (1951).
3. Bernstein, E. K., S. P. Stearner, and A. M. Brues. Studies of liver function in the chick following X-irradiation: Plasma amino acids and plasma glucose. *Am. J. Physiol.* 186:543-548 (1956).
4. Stearner, S. P. Unpublished data.
5. Stearner, S. P., M. Sanderson, E. J. Christian, and A. M. Brues. Initial radiation syndrome in the adult chicken. *Am. J. Physiol.* 184:134-140 (1956).
6. Stearner, S. P., M. Sanderson, and E. J. Christian. Progress report: Radiation studies in birds. The red cell mass of peripheral blood and other tissues of normal and X-irradiated roosters.. Quarterly Report of Biological and Medical Research Division, Argonne National Laboratory. ANL-5597, pp. 21-24 (1956).

PROGRESS REPORT: THE BIOSYNTHESIS OF METHIONINE IN BACTERIA

A New Transmethylation

Stanley K. Shapiro

The methylation of homocysteine with S-methylmethionine as the methyl donor has been reported previously. (1, 2, 3, 4) It was shown that S-adenosyl-DL-methionine was slightly active as a methyl donor. However, it has now been found possible to isolate pure S-adenosyl-L-methionine from yeast. (5) With this compound it was found that transmethylation of homocysteine occurs readily with enzymes prepared from Aerobacter aerogenes. A series of results is shown in Table 23. The analytical methods used have been described in detail previously. (4) It can be seen that 40% of the substrate was utilized for transmethylation. S-adenosylmethionine when incubated alone with the enzyme mixture is readily decomposed into various breakdown products. This, of course, depletes the amount of methyl donor remaining for the transmethylase activity of the extract. S-adenosyl-D-methionine is relatively inactive as a methyl donor.

TABLE 23

The enzymatic synthesis of methionine from
homocysteine and S-adenosyl-L-methionine
with extracts of Aerobacter aerogenes
(incubated for 2 hr at 35°C)

Substrate (4 μ M/ml)	Methionine formed, μ M/ml
None	0
S-adenosyl-L-methionine	0
DL-homocysteine	0
S-adenosyl-L-methionine + DL-homocysteine	1.6

Active homocysteine transmethylases have also been prepared from cells of Torulopsis utilis and Saccharomyces cerevisiae. These yeast extracts as well as the bacterial extracts can utilize S-methylmethionine as well as S-adenosyl-L-methionine as methyl donors for the transmethylation of homocysteine. It will be important to unravel the relationship between the methyl donors and to determine whether there are separate enzyme systems involved.

References

1. Shapiro, S. K. The biosynthesis of methionine from homocysteine and methylmethionine sulfonium salt. Report on Biological, Medical and Biophysics Program. Argonne National Laboratory. ANL-5456, pp. 58-60 (1955).
2. Shapiro, S. K. The biosynthesis of methionine in normal bacteria and in ultraviolet-induced methionine mutants. Quarterly Report of Biological and Medical Research Division, Argonne National Laboratory. ANL-5486, pp. 35-36 (1955).
3. Shapiro, S. K. The methylation of homocysteine to methionine in bacteria. Quarterly Report of Biological and Medical Research Division, Argonne National Laboratory. ANL-5576, pp. 28-29 (1956).
4. Shapiro, S. K. Biosynthesis of methionine from homocysteine and S-methylmethionine in bacteria. J. Bacteriol. In press (1956).
5. Schlenk, F., and R. E. DePalma. Progress report: The metabolic relation between adenine and sulfur amino acids. Isolation of S-adenosylmethionine from yeast. Report on Biological, Medical and Biophysics Programs, Argonne National Laboratory. ANL-5518, pp. 134-139 (1956).

GLYCOGEN PARTICLES IN RAT LIVER HOMOGENATES

A. D. Barton and Anna Kane Laird

A previous report⁽¹⁾ described experiments dealing with the effects of sodium desoxycholate (DC) on the particles in the microsome fraction of rat liver. It was shown that since this compound lyses the other components of the microsome fraction^(2, 3) it provides a convenient means for isolating the glycogen particles. The isolation and characterization of these particles is described more fully in the present report.

In a typical experiment the glycogen particles were prepared as follows for study in the electron microscope. A 10% homogenate of liver, either in 0.88 M sucrose or in water, was centrifuged at 13,000 $\times g$ for 15 min in order to remove mitochondria, nuclei, unbroken cells, etc. The glycogen particles vary widely in size, and are not readily resuspended to yield the original particles after they have been consolidated by excessive centrifugation. Consequently, samples of the supernatant, after being mixed with an equal volume of 2% DC, were centrifuged at graded gravitational forces, the supernatants were discarded, and the parts of the sediments that resuspended easily were taken for examination. The suspensions were diluted extensively with distilled water, deposited on grids, dried, and shadowed with palladium.

A typical preparation is shown in Figure 24. As a result of the DC treatment, this preparation consisted almost entirely of glycogen and contained only very small amounts of nitrogen and phosphorus; the glycogen appears in the form of dome-shaped bodies having low electron density. Figure 25 shows a comparable microsome preparation that was not treated with DC. The glycogen particles are distinguished easily from the other constituents of the microsome fraction, since their electron density is much less than that of the fragments of the endoplasmic reticulum or of the small ribonucleoprotein granules. The large glycogen bodies of irregular shape probably consist of material that was compacted by the sedimentation and not resuspended adequately. The round bodies apparently are particles that were present in the homogenate, since the range of sizes observed is not altered by the treatment with DC, or by further extensive dilution of the suspension.

In various preparations, the round glycogen particles range in size from about 2500 Å down to about 200 Å, in which range it becomes difficult to distinguish them with certainty from the ribonucleoprotein granules. If, for purposes of calculation, the glycogen particle in these dried preparations is viewed as the top portion of a sphere, having a thickness approximately equal to one-tenth of its diameter, then such a particle, 2500 Å in diameter will have a volume equal to that of a sphere about 1250 Å in diameter.

Figure 24. Glycogen particles, obtained by treating the microsome fraction of rat liver with sodium desoxycholate. Glycogen particles, g; fragments of endoplasmic reticulum, f; ribonucleoprotein granules, n. The reference length shown is 1μ .

Figure 25. Particles of the microsome fraction not treated with sodium desoxycholate. Symbols and reference length are the same as those used in Figure 24.

The classical procedures for the isolation and purification of glycogen involve digestion of the tissue with hot concentrated alkali or precipitation with trichloroacetic acid (TCA). Such preparations have yielded glycogen with molecular weights in the range $2-10 \times 10^6$,⁽⁴⁾ and their partial specific volume has been found to be 0.65.⁽⁴⁾ On this basis, the particles of this material would be 100-150 A in diameter, i. e., about one-tenth of the diameter of the largest glycogen particles in the homogenate. In the present study, these dimensions have been confirmed by examination of such preparations in the electron microscope.

Sedimentation of the glycogen of rat liver has been compared with that of purified glycogen. Samples of rat liver homogenate, or of glycogen isolated after digestion of the tissue with hot concentrated alkali were suspended in distilled water, in aqueous DC at a final concentration of 1%, or in aqueous TCA, at a final concentration of 5%. Each suspension was sedimented first at $13,000 \times g$ for 15 min, and the supernatant was then sedimented at $19,000 \times g$ for 15 min. Each of the sediments and final supernatants was analysed for glycogen by the anthrone method.

The results are summarized in Table 24. In confirmation of earlier work, the "particulate" glycogen of the homogenate sediments readily; the purified glycogen does not.^(5, 6) Although the DC treatment lyses many of the particles in the homogenate, it has little effect on the sedimentability of the glycogen, whereas the latter is drastically reduced by the TCA.

TABLE 24

Sedimentation of glycogen in liver homogenate
and of glycogen purified with alkali

	Sediment (13000 $\times$ g)	Sediment (19000 $\times$ g)	Supernatant (19000 $\times$ g)
Homogenate	25	21	54
Homogenate + DC	18	21	62
Homogenate + TCA	8	7	86
Glycogen	1	4	95
Glycogen + DC	1	2	97
Glycogen + TCA	0	2	98

Results are expressed as a percentage of the total glycogen present in the original homogenate or glycogen solution.

The results with the electron microscope, as well as the sedimentation data, indicate that the glycogen of the homogenate is not included in other bodies but exists separately in particles showing a wide range of sizes, the largest of which may have ten times the diameter and hence one thousand times the volume of a glycogen molecule. Disruption of these large glycogen particles by TCA suggests that the small amount of protein usually found with glycogen may play a structural role in these particles.

References

1. Barton, A. D., and A. K. Laird. Morphological and biochemical effects of sodium desoxycholate on the microsome fraction of rat liver. Quarterly Report of Biological and Medical Research Division, Argonne National Laboratory. ANL-5518, pp. 151-153 (1956).
2. Littlefield, J. W., E. B. Keller, J. Gross, and P. C. Zamecnik. Studies on cytoplasmic ribonucleoprotein particles from the liver of the rat. J. Biol. Chem. 217: 111-123 (1955).
3. Palade, G. E., and P. Siekevitz. Liver microsomes. An integrated morphological and biochemical study. J. Biophys. Biochem. Cytol. 2: 171-200 (1956).
4. Bell, D. J., H. Gutfreund, R. Cecil, and A. G. Ogston. Physicochemical observations on some glycogens. Biochem. J. 42: 405-408 (1948).
5. Lazarow, A. Particulate glycogen: A submicroscopic component of the guinea pig liver cell; its significance in glycogen storage and the regulation of blood sugar. Anat. Record 84: 31-50 (1942).
6. Claude, A. Fractionation of mammalian liver cells by differential centrifugation. J. Exptl. Med. 84: 51-98 (1946).

PROGRESS REPORT: PROTEIN SYNTHESIS IN PANCREAS

III. Subfractionation of microsomes

Anna Kane Laird and A. D. Barton

In a recent series of cell fractionations of pancreas,⁽¹⁾ it was shown that a large part of the amylase recovered was found in the microsome and final supernatant fluid fractions, and that the distribution between the two fractions varied with the secretory activity of the gland.

More recently, a report has appeared of the demonstration in dog pancreas of a significant proportion of amylase and other digestive enzymes in the white pellet which underlies the mitochondrial sediment.⁽²⁾ Morphologically, the particles of the white pellet consist of small, round, sharply defined bodies, about 1 to 2 μ in diameter; they are undoubtedly the secretory granules of classical histology. While our assay of this material in rat pancreas showed that the highest specific activity of amylase with respect to protein-nitrogen is found in the secretory granules (Table 25), nevertheless at least 50% of the enzyme activity is recovered elsewhere in rat pancreas, even if we assume that all the enzyme activity missing after the prolonged fractionation procedure is derived from the secretory granules.

TABLE 25

Biochemical properties* of secretory granules, microsomes, and supernatant fluid of rat pancreas

Experiment †	Amylase**		RNA or E ₂₆₀		Phospholipid	
	1	2	1	2	1	2
Secretory Granules	4.3	5.3		.0005	-	.027
Microsomes (from 0.25 M sucrose)	2.3	1.3	1.6	.0008	.07	.050
Supernatant Fluid	1.6	1.3	0.89	.0001	.031	.011

*Activities and concentrations relative to protein-nitrogen.

**Amylase units: E₅₃₀/γN x 10⁻³.

† Experiment 1: RNA determined by the orcinol reaction;
supernatant fluid obtained in 0.88 M sucrose;
Experiment 2: E₂₆₀ read directly on suspension;
supernatant fluid obtained in 0.25 M sucrose;

In order to study in more detail the extragranular amylase, the microsome fraction has been subjected to two types of subfractionation procedure: in one, the centrifugal force and time were kept constant while the viscosity (and osmotic properties) of the medium were varied; in the other, the medium was kept constant, and the centrifugal force was varied. The results of the former type of procedure are shown in Table 26. As would be expected, higher proportions of all the constituents studied were sedimented when the viscosity of the medium was reduced, and especially if the pH was also lowered to 5 (Table 26 A). The composition of the larger pellets thus obtained was similar to that obtained in 0.88 M sucrose at neutral pH (Table 26), although it is evident that reducing the pH has resulted in the sedimentation of additional protein which is not associated with RNA, phospholipid, or amylase activity, since the ratio of all three constituents to protein-nitrogen in the pH 5 sediment was reduced in comparison with the otherwise comparable sediment obtained from isotonic sucrose at neutral pH.

TABLE 26

Biochemical properties of microsome pellets sedimented from different media at 100,000 x g for 60 min

	Amylase*	RNA**	Phospholipid	Protein-nitrogen
A. Quantities sedimented:				
0.88 <u>M</u> sucrose	11.0	4.13	.207	2.76
0.25 <u>M</u> sucrose	15.9	5.49	.255	3.52
0.25 <u>M</u> sucrose, pH 5	19.4	10.0	.446	8.52
B. Concentrations relative to protein-nitrogen:				
0.88 <u>M</u> sucrose	3.96	1.50	.075	
0.25 <u>M</u> sucrose	4.44	1.56	.072	
0.25 <u>M</u> sucrose, pH 5	2.27	1.17	.052	
0.25 <u>M</u> sucrose, pH 5	0.51	0.75	0.73	
0.25 <u>M</u> sucrose				

*mg x 10⁴ maltose produced in 30 min per gram equivalent of pancreas.

**mg per gram equivalent of pancreas.

Entirely different results were obtained when an isotonic suspension of microsomes was subjected to increasing centrifugal force (Table 27). In three experiments the results were essentially the same in spite of differences in the condition of the animals and in the preparation of the homogenate.

TABLE 27

Biochemical properties of microsome pellets prepared by differential centrifugation

	Amylase		RNA or E ₂₆₀		Phospholipid		Protein-nitrogen
	% of total	Rel. activity*	% of total	Rel. conc'n.**	% of total	Rel. conc'n.	% of total
			Experiment 1				
25,000 x g	33	1.3	30	.22 [†]	67	10	25
75,000 x g	22	1.1	15	.31 [†]	17	.04	15
100,000 x g	12	0.52	15	.14 [†]	11	.01	30
			Experiment 2				
25,000 x g	53	.47	45	1.4	81	.11	42
75,000 x g	11	.39	17	2.1	10	.05	11
100,000 x g	7	.34	14	2.5	1	.01	8
			Experiment 3				
25,000 x g	56	.49	46	1.5	83	.11	40
75,000 x g	8	.36	13	2.0	8	.05	8
100,000 x g	11	.31	22	2.2	6	.02	13

*E₅₃₀ per γ protein-nitrogen x 10⁻³.

**mg RNA per mg protein-nitrogen, with exceptions noted.

[†]E₂₆₀ per γ protein-nitrogen.

In the first experiment the animals were starved overnight, with the result that both the total quantity of amylase and the proportion of the amylase in the supernatant fluid are higher than in the last two experiments given, in which the animals were allowed to feed ad libitum overnight (see reference 1). In both the first and second experiments, the pancreas was homogenized in 0.88 M sucrose, with subsequent dilution of the mitochondrial supernatant to bring the sucrose concentration to 0.25 M before sedimentation

of the microsomes, while in the third experiment the pancreas was homogenized in 0.25 M sucrose. In all three experiments, striking differences were noted in the various subfractions obtained. A 5- to 10-fold difference in the ratio of phospholipid to protein-nitrogen was observed between the sediment obtained at 25,000 x g and that obtained at 100,000 x g; 1.5- to 2-fold differences in the ratio of RNA to protein-nitrogen and about a 1.5-fold difference in the ratio of amylase to protein-nitrogen were also found between these sediments. The considerable differences between the subfractions produced by varying the centrifugal force are in clear contrast to the close similarities in the biochemical properties of the subfractions obtained by varying the viscosity of the medium.

These results show that, in a system such as this, varying the viscosity of the medium does not produce the same effects as varying the centrifugal force. In varying the viscosity of the medium we are also varying the osmotic pressure to which the particles are subjected, and it is known that the "endoplasmic reticulum" elements of the microsome fraction respond to changes in osmotic pressure with considerable changes in shape and volume.⁽³⁾ Although the microsome fraction consists chiefly of fragments of endoplasmic reticulum, it does contain other bodies, and it is possible that these various elements respond differently to changes in osmotic pressure.

References

1. Laird, A. K. and A. D. Barton. Progress report: Protein synthesis in pancreas. Intracellular localization of a pancreatic digestive enzyme. Quarterly Report of Biological and Medical Research Division, Argonne National Laboratory. ANL-5518, pp. 131-133 (1956).
2. Hokin, L. E. Isolation of zymogen granules of dog pancreas and a study of their properties. *Biochim. et Biophys. Acta* 18: 379-388 (1955).
3. Palade, G. E. and P. Siekevitz. Liver microsomes. An integrated morphological and biochemical study. *J. Biophys. Biochem. Cytol.* 2: 171-200 (1956).

ORGANELLE PATTERNS OF THE CELL BORDER IN PARAMECIUM

Charles F. Ehret and E. L. Powers

Fragments of the cell border or pellicle in Paramecium bursaria are readily separated from the living cell by means of sudden compression-decompression between slide and cover slip. When these fragments are examined with the phase contrast microscope they appear to consist entirely of close-packed corpuscular organelles. Two systems are evident: (a) a generalized pellicle system, and (b) a specialized food intake or gullet system. The first is sheet-like, with hexagonal and rhomboidal packing patterns; its organelles are about 1.8μ in diameter, and these may float freely away from the edge of a dissected sheet as spheroids with their cilia and kinetodesmal fibrils still intact. The gullet system is tube-like, and contains three organelle-complexes: peniculus (8 columns of "granules," and cilia), quadrulus (4 columns of "granules," and cilia), and ribbed-wall (≥ 20 columns of "granules" but no cilia). The dot-like "granules" of the gullet-complexes are each about 0.25μ in diameter; they are close-packed in peniculus and ribbed wall, and have a frequency about one-fourth that of the pellicle-system organelles.

Electron micrographs of longitudinal, tranverse and tangential sections of paramecia substantiate the existence of corpuscular organelles at the cell border. Furthermore, the classical patterns of the so-called "fibrillar systems" are all evident in such sections, and the "silver-lines," Schuberg lines,⁽¹⁾ and Gabor von Gelei patterns⁽²⁾ are all derived not from fibers but from sheets and surfaces of the basic packing units. The bona fide fibrils remaining are those which compose the kinetodesma.⁽³⁾

We conclude that over the past 50 years as a consequence of optical sectioning,⁽²⁾ selective deposition of stains^(1,2) and disruption of elements,⁽³⁾ portions of actual sheets and surfaces have been termed "fibrils." This is not to say that the ultrastructure of these surfaces may not involve fibrous proteins, but simply that to call the edge of a sheet a "fibril" is a misinterpretation. With the fibrillar lattices eliminated, the old questions regarding their interconnectives seem pointless: the cilium and kinetodesmal fibril appear to be integral components of a corpuscular organelle that constitutes a primary packing unit for the complexes and systems of the free cell border.

References

1. Schuberg, A. Über Cilien und Trichocysten einiger Infusorien. Arch. Protistenk. 6: 61-110 (1905).
2. Gelei, G. von. Ein neues Fibrillensystem im Ectoplasma von Paramecium. Arch. Protistenk. 89: 133-162 (1937).
3. Metz, C. W., D. R. Pitelka and J. A. Westfall. The fibrillar systems of ciliates as revealed by the electron microscope. I. Paramecium. Biol. Bull. 104: 408-425 (1953).

ELECTRON MICROSCOPIC INVESTIGATION OF THE WALL OF THE BLOOD CAPILLARIES OF THE LIVER

F. Wassermann

The relationship between the blood capillaries of the liver, called sinusoids, and the hepatic cells is beginning to be understood with the aid of the electron microscope. Up to the present time no definite knowledge has been obtained about the walls of the sinusoids, i.e., whether they consist of a continuous or discontinuous layer of endothelial cells, nor about the phagocytic Kupffer cells, i.e., whether they are ordinary endothelial cells or a special type of cell. Controversial discussions concern also the space of Disse between the walls of the sinusoids and those of the "lacunae" which are formed by the hepatic cells (see Elias).⁽¹⁾ Fawcett⁽²⁾ was the first to undertake an electron microscopic study of these structures, with the remarkable result of discovering what he termed the villi of the liver cells. They are shown in the pictures presented in this report. However, Fawcett's observations covered only part of the structures which are pertinent to the above-mentioned questions, and the picture given in his paper of the wall of the sinusoids does not, according to our own findings, represent the most frequently encountered situation. Fawcett did not demonstrate endothelial cells or Kupffer cells and did not look for the reticulin fibers in the wall of the sinusoids.

When, in the course of our electron microscopic studies of collagenous microfibrils, our interest turned to the reticulin or argyrophilic fibers of the liver, which have not been demonstrated before in electron microscopic sections, it became evident that our observations will allow a more complete description of the structures of the wall of the sinusoids than has been given hitherto.

Small pieces of the livers of albino rats were fixed in buffered osmic acid (pH 7.5) for 1 hr, dehydrated, without washing, in 50% to 100% ethyl alcohol, and imbedded in methacrylate. Sections of about 400 m μ were cut with the Porter-Blum microtome and studied with the RCA EMU-2A and EMU-3A electron microscopes.

The results obtained so far can be summarized as follows:

1. In the majority of our electron micrographs the wall of the sinusoid was found as shown in Figure 26 A, to consist of a lining (li) which seems to close the space of Disse (D). Interruptions of the lining occur frequently. Some of them are undoubtedly artifacts due to fixation or to the cutting knife. Several sections gave evidence that the lining, which is a very delicate structure, can be easily destroyed and therefore may be missing entirely. Whether or not true discontinuities of the lining exist, remains to be seen.

- A. The picture shows the lumen of a sinusoid containing an erythrocyte. Notice the lining of the sinusoid and the space of Disse between the lining and the hepatic cells which is occupied by the "villi" of the hepatic cells.

- B. The picture shows an endothelial cell resting upon the villi of the hepatic cell. Notice that the cytoplasm of the endothelial cell continues into the lining of the sinusoid.

Figure 26.

hc - hepatic cells	li - lining of the sinusoid
s - sinusoid	en - endothelial cell
e - erythrocyte	n - nucleus
D - space of Disse	K - Kupffer cell
v - villi of the hepatic cells	rf - reticulin fibers

- C. The picture shows part of the lumen of a sinusoid with an erythrocyte. A Kupffer cell protrudes into the lumen. It is closely attached to the hepatic cell in part by being in touch with the villi, in part by processes which extend into indentations of the surface of the liver cell (arrow).

- D. The picture shows part of the lumen of a sinusoid. Beneath the lining of the sinusoid within the space of Disse a reticulin fiber, consisting of cross-banded microfibrils is seen.

Figure 26 (Cont'd.)

2. The lining is, as Figure 26 B clearly shows, formed by the cytoplasm of the endothelial cells. The latter are characterized by a nucleus which differs from that of the liver cells by unusually large and dense chromocenters. It is surrounded by a small area of cytoplasm. On the side adjacent to the hepatic cells the endothelial cells are closely attached to the villi.

3. The Kupffer cells (Figure 26 C) are larger than the flat endothelial cells but have the same kind of nucleus and show an even more intimate relationship to the liver cells. The protoplasmic processes of the Kupffer cells are in close touch with the villi, and it is often impossible to find a border between them. Also the many interlockings between Kupffer cells and hepatic cell (arrows, Figure 26 C) are of interest. Although some of our pictures strongly suggest that Kupffer cells may be liberated and carried away by the blood stream as macrophages, more observations especially on cells labelled by injection of the animal with India ink are needed before a definite statement can be made about this point.

4. The reticulin fibers of the liver (Figure 26 D) are found within the space occupied by the villi beneath the lining of the sinusoids. They consist of loosely aggregated microfibrils having widths of the order of about 300 A. The microfibrils show a characteristic spacing along the axis. Although in the photograms so far obtained the length of the periods could not be determined accurately, it seems to lie within the range of the periodicity of collagen.

References

1. Elias, H. Liver morphology. Biol. Rev. 30:263-310 (1955).
2. Fawcett, D. W. Observations on the cytology and electron microscopy of hepatic cells. J. Natl. Cancer Inst. 15:1475-1502 (Supplement) (1955).

PROGRESS REPORT: DOSE RATE EFFECT UPON TERATOGENESIS IN GRASSHOPPER DEVELOPMENT

Theodore N. Tahmisian and Betty Jean Wright

Evans⁽¹⁾ has described X-ray-induced teratogenesis in the grasshopper (Melanoplus differentialis) embryo during prediapause development. He reported that most of the abnormalities in organization and regulation occurred when the embryos were irradiated on the 6th and 7th days of prediapause development, and that a dose of 200 r to 400 r was optimal.

Using the above information we undertook the study of the effect of dose rate upon teratogenesis in M. differentialis embryos.

Materials and Methods

Grasshoppers in the colony were provided with small dishes of sand. As soon as a female began oviposition the dish of sand with the laying grasshopper was removed from the cage and the pod was collected, thus enabling the age of the eggs to be precisely determined. The eggs were allowed to develop in the pods for 5 or 7 days, at which time samples from each end of each pod were dissected to insure that the pods were of the required age according to Slifer.⁽²⁾ Pods containing eggs that were older or younger than desired were discarded and the remaining pods were then pooled. One group was irradiated with 250 r at 200 r/min (250 kv, 15 ma), a second with 250 r at 1 r/min (250 kv, 2 ma), and a third group was kept as a control. At various days following irradiation, a number of eggs from each group were dissected and the abnormalities were observed with the aid of a dissecting microscope.

Results and Discussion

Abnormalities consisted of deletions, disjunctions, repetitions, stunts, and bifurcations (Figure 27). However, the actual types of injury and their implications with respect to axial gradients were not considered in scoring the results. The total number of deformed embryos was noted, and the deformities were classified as to the region (head, thorax, or abdomen) on which they occurred.

In the eggs irradiated on the 7th day of prediapause development, 76% of the embryos exposed at 200 r/min showed abnormalities, while only 38% of those exposed at 1 r/min were abnormal (Table 28). The percentage of deformities was highest in the abdominal region and lowest in the head. There was no difference in the types of abnormalities produced at the two dose levels. However, since more embryos were normal after irradiation at the lower rate, it appears either that the animal is capable of recovery from, or that it fails to react to, a previously effective dose administered at a lower rate. Similar results were obtained in the two replications making up this experiment.

Figure 27. Abnormalities observed in grasshopper embryos exposed to 250 r of X-radiation at low and high dose rates.

- A. Control embryos, 20-day prediapause.
- B. Irradiated at 200 r/min on the 7th day of prediapause development. Dissected and photographed 12 days postirradiation. Upper embryo shows bifurcated antennae. Lower embryo shows abdominal deletion, double head, and stunted appendages.
- C. Irradiated at 1 r/min on the 7th day of prediapause development. Dissected and photographed 12 days postirradiation. Upper embryo has bifurcated antennae and appendages. Lower embryo has three compound eyes and a thin abdomen.
- D. Irradiated at 200 r/min on the 7th day of prediapause development. Dissected and photographed 14 days postirradiation. Embryo shows double head, supernumerary mouth parts, and stunted mouth parts.

Figure 27 (Cont'd.)

- E. Irradiated at 200 r/min on the 7th day of predia-pause development. Dissected and photographed 14 days postirradiation. Embryo shows abdominal deletion, deranged thoracic appendages, a double stomodeum, and a double head with extra mouth parts.
- F. Irradiated at 200 r/min on the 5th day of predia-pause development. Dissected and photographed 13 days postirradiation. Attached below the 6th abdominal segment of the embryo is a second embryo with deranged appendages.
- G. Irradiated at 200 r/min on the 5th day of predia-pause development. Dissected and photographed 13 days postirradiation. The abdomen has double the normal number of segments.
- H. Irradiated at 1 r/min on the 5th day of predia-pause development. Dissected and photographed 13 days postirradiation. Embryo has a completely disorganized abdomen growing in different directions. The head is asymmetrical and has large mouth parts. Appendages are fused.

TABLE 28

Abnormalities observed in grasshopper embryos exposed to 250 r of X-rays on the 7th day of development

	200 r/min			1 r/min		
	Number of observations	Deformed		Number of observations	Deformed	
		Number	%		Number	%
Entire embryo	166	126	76	165	62	38
Head	166	64	39	165	20	12
Thorax	166	55	33	165	14	8
Abdomen	166	92	55	165	53	32

In embryos irradiated on the 5th day of development, the difference in response to high and low dose rates was not so great. Of those irradiated at 200 r/min, 78% were abnormal, while of those irradiated at 1 r/min, 61% were abnormal (Table 29). In all cases the 5-day embryos showed a higher response to 1 r/min in comparison to the 7-day embryos receiving 1 r/min. The 5-day embryos showed many more thoracic abnormalities than did the 7-day embryos, regardless of dose rate, and this increase was proportionately greater than those for head or abdomen. This indicates that induction or determination occurs earlier in the thorax than in the other two areas.

TABLE 29

Abnormalities observed in grasshopper embryos exposed to 250 r of X-rays on the 5th day of development

	200 r/min			1 r/min		
	Number of observations	Deformed		Number of observations	Deformed	
		Number	%		Number	%
Entire embryo	105	82	78	106	65	61
Head	105	50	48	106	32	30
Thorax	105	61	58	106	41	39
Abdomen	105	62	59	106	53	50

Since all of the embryos continued to grow after irradiation, it appears that cell division is not affected, either at high or low exposure rates, by a dose sufficient to cause abnormalities in organization and development. It also appears that for a given dose, the degree of teratogenic effect increases with the dose rate.

The time of tissue induction appears to be spread over a period of at least 3 days, supporting the conclusion of Evans⁽¹⁾ that 3 days is the total time involved. However, since the thorax evidently is determined earlier in the development of the embryo, its time of induction must either fall within an earlier 3-day period or cover a broader time range.

Further experiments are contemplated to determine whether radiation effects on induction are primarily a function of dose rate or of the total period of time of irradiation.

References

1. Evans, T. C. Effects of roentgen radiation on embryonic organization and regulation in Melanoplus differentialis (Orthoptera). *Physiol. Zool.* 10: 258-267 (1937).
2. Slifer, E. H. Insect development IV. External morphology of grasshopper embryos of known age and with a known temperature history *J. Morphol.* 53: 1-22 (1932).

PROGRESS REPORT: SURVIVAL OF LAF₁ MICE GIVEN DAILY DOSES OF GAMMA RAYS

George A. Sacher, Douglas Grahn and Katherine F. Hamilton

The recruitment of mice into the duration-of-life exposure condition is well advanced, and completed survival records have passed the halfway point in numbers. However, the data at the lowest treatment levels are somewhat less complete.

Table 30 gives mean after-survivals and standard deviations for male and female LAF₁ mice exposed at levels from 12 to 220 r/day. These data are based on completed replications. Thus the mean after-survivals are not seriously biased even though they are based on incomplete results.

Comparison of the two sexes reveals that there is a reversal of their relative radiosensitivities as survival times increase. Males are more resistant than females at dosage levels that yield survival times less than 40 days, that is, at 125 r/day and above. In the range from 97 down to 74 r/day (survival times of 40 to 70 days) there is no significant sex difference, and at all lower dosage levels the female mouse shows better survival. The superiority of the female becomes more evident as survival time increases. At 12 r/day there is a 100-day difference in the survival of the two sexes. Data on incomplete replications at 6 and 0 r/day suggest that the difference between sexes at these dose rates will not be so great as those observed at 12 r/day.

Sex differences are also observable in the magnitudes of the standard deviations (Table 30). The differences in standard deviations seem to a considerable extent to be independent of the sex differences in mean after-survival. This suggests that the study of sex differences in lethality will reveal the existence of 2 or more independent mechanisms of lethality in the subacute and chronic regions.

The overall relation between daily dose and mean survival time (Figure 28) conforms to the pattern established for the mouse by previous work in this laboratory and elsewhere.⁽¹⁾ A disturbance in the relation at survival times in the neighborhood of 70-140 days (daily doses of 49-64 r) will require careful study.

Survivorship curves for the combined sexes at daily dosages from 97 to 32 r are presented in Figure 29. The nonuniform progression of survival times at dosage levels from 49 to 64 r/day is quite evident in this mode of presentation. Also of interest is the change in the shape of the survival curve between 49 and 43 r/day. In view of the sample sizes, these irregularities are probably significant.

TABLE 30

Mean after-survival (MAS) and standard deviations (σ) for
LAF₁ mice irradiated daily for the duration of life,
beginning at 100 days of age

Daily dose, r	Sex	n	MAS	σ^2	σ	Daily dose, r	Sex	n	MAS	σ^2	σ
12	M	27	312.30	16391.3703	128.03	74	M	90	64.98	316.0220	17.78
	F	27	412.18	9225.0028	96.05		F	90	65.65	192.1384	13.86
	M+F	54	362.24	15108.0353	122.91		M+F	180	65.32	252.7763	15.90
24	M	66	223.41	7119.8762	84.38	85	M	60	52.80	188.7729	13.74
	F	66	263.64	6073.3734	77.93		F	60	51.12	157.1588	12.54
	M+F	132	243.52	6953.9155	83.39		M+F	120	51.96	133.2672	11.54
32	M	60	193.98	3322.7751	57.64	97	M	89	41.90	30.2510	5.500
	F	60	205.02	3089.2709	55.58		F	90	41.96	37.8856	6.155
	M+F	120	199.48	3210.0498	56.66		M+F	179	41.93	33.8992	5.822
43	M	75	144.04	1430.2281	37.82	125	M	105	33.88	21.5830	4.646
	F	75	153.13	2150.3604	46.37		F	105	31.17	28.2396	5.314
	M+F	150	148.59	1799.0898	42.41		M+F	210	32.53	26.6427	5.162
49	M	45	126.76	460.4616	21.46	145	M	60	28.63	30.5751	5.530
	F	45	137.22	871.6768	29.52		F	60	24.52	13.8811	3.726
	M+F	90	131.99	686.2808	26.20		M+F	120	26.58	26.3136	5.130
56	M	75	95.07	472.0901	21.73	170	M	90	22.34	18.5429	4.306
	F	75	99.43	492.5993	22.19		F	90	20.19	6.9414	2.635
	M+F	150	97.25	483.8918	22.00		M+F	180	21.27	13.8391	3.720
64	M	45	84.84	405.8616	20.15	220	M	75	16.47	3.4144	1.848
	F	45	95.49	382.2101	19.55		F	75	15.81	3.5322	1.879
	M+F	90	90.17	418.2528	20.45		M+F	150	16.14	3.5574	1.886

Figure 28. Relation between mean after-survival of LAF₁ mice and size of daily dose. Data at dosage levels of 220 r daily and below from Table 30. Data at higher levels reported previously. The dashed line has a slope of -1 and the dotted line has a slope of -2. They are provided for visual comparison.

Figure 29. Survivorship curves for LAF₁ mice exposed to daily dosages in the range 97-32 r. Each curve is accompanied by the daily dosage and (in parentheses) the number of mice. There were equal numbers of the two sexes.

We are indebted to Miss Joan Gurian for the supervision of IBM computations.

Reference

1. Sacher, G. A. A comparative analysis of radiation lethality in mammals exposed at constant average intensity for the duration of life. J. Natl. Cancer Inst. 15: 1125-1140 (1955).

ACUTE LETHAL RESPONSE OF FIRST AND SECOND GENERATION HYBRID MICE TO WHOLE-BODY X-IRRADIATION

Douglas Grahn and Katherine Hamilton

Previous reports^(1,2) have indicated the extent of genetic variation in the acute lethal response of four inbred mouse strains to whole-body X-irradiation. In order to study the mode of inheritance of this variation in radiosensitivity, the response of the progeny of crosses between genetically different inbreds must be evaluated. The most sensitive strain, BALB/c, with an LD₅₀ of 500 r, and the most resistant strain, C57BL/6, with an LD₅₀ of 630 r, have been reciprocally crossed to produce the F₁ hybrid generation. An F₂, or segregating generation, has been produced by breeding F₁ mice inter se. Factors pertinent to the selection and irradiation of animals, as well as the statistical analysis of the data, are the same as those used for the parent inbreds and have been described in detail by Grahn and Hamilton.⁽³⁾ All LD₅₀ values are based on a constant age at exposure of 85 days. A total of 896 F₁ and 691 F₂ mice were irradiated.

First generation hybrids (F₁): The LD₅₀ value is estimated to be 659.5 ± 6.6 r with a dosage-arcsine mortality slope of $+ .404 \pm .028$. A breakdown of the F₁ mice into sex and reciprocal groups is given in Table 31. Dosage-mortality slope estimates were virtually identical for all comparisons and consequently are not given.

TABLE 31

LD_{50/30} values by sex and reciprocal for BALB/c : C57BL/6 F₁ hybrids

Reciprocal cross	♀	♂	Combined sexes
CB (BALB ♀ x C57BL ♂)F ₁	676.4 r	648.3	661.6 r
BC (C57BL ♀ x BALB ♂)F ₁	675.8 r	654.1	662.4 r
Combined reciprocals	676.7 ± 7.0 r	651.5 ± 5.3 r	

A significant sex difference ($P < .025$) is apparent, the female having an LD₅₀ value 25 r higher than the male. This sex difference is consistent with the findings in the parent inbreds,⁽³⁾ but of greater magnitude.

The two reciprocal crosses produced mice of equal sensitivity, indicating an absence of maternal influence. Sex differences, within reciprocal, are the same as for the combined estimate for the sexes, showing that sex-linked genes are not significantly involved in the difference between the two parental strains.

Second generation hybrids (F_2). The LD_{50} value for the F_2 mice is estimated to be 627.8 ± 6.6 r. The dosage-mortality slope is $+ .274 \pm .026$. Peculiarly, the significant sex difference noted in the F_1 hybrids all but disappears in the F_2 . The LD_{50} for the females is 631.9 r and that for the males is 629.3 r.

Since the F_1 mice are heterozygous for agouti, black and albino (AaBbCc), the F_2 generation segregates five coat color types. These are black agouti, brown agouti, black, brown and albino in an expected ratio of 27:9:9:3:16, respectively. Nonagouti, black and full color are derived from the C57BL parent, while agouti, brown and albino come from the BALB parent. The F_2 data have been subdivided into color types in order to examine the possibility of any nonrandom segregation of sensitivity factors with the color genes. Comparisons are made on an alternative gene basis so as to maximize sample sizes. The results are given in Table 32. While the agouti and black loci show only minor differences, the albino locus presents a significant difference between the two alternatives.

TABLE 32

$LD_{50/30}$ values for coat color comparisons in BALB/c : C57BL/6 F_2 mice.

Number of mice of given color type shown in parentheses

BALB/c contribution		C57BL/6 contribution	Difference
Agouti (384)	versus	Nonagouti (134)	
640.1 r		635.5 r	4.6 r
Brown (157)	versus	Black (361)	
635.6 r		642.0 r	6.4 r
Albino (173)	versus	Nonalbino (518)	
602.3 ± 8.6 r		639.3 ± 7.9 r	37.0 ± 11.7 r
			$P < .01$

Several hypotheses can be advanced to explain the genetic mechanism underlying the sensitivity of the albino. One, it may be due to physiologic manifestations of the albino gene specifically, or, two it may be due to genes closely linked to the albino locus. It cannot, however, be

attributed to any natural selection factors acting prior to test that could have eliminated potentially sensitive mice from the nonalbino class, because the expected 3:1 ratio was observed to a perfect degree. Of the 691 mice tested, the observed ratio was 518:173; the expected would be 518.25:172.75. Since all available F_2 mice were employed in this test series, the ratio observed is the actual segregation ratio measured at an average age of 85 days.

Confirmation of the greater radiosensitivity of the albino was obtained from a small group of test-cross mice derived from the mating of the F_1 female with the A/Jax male; the latter being the homozygous recessive (aabbcc, nonagouti, brown, albino) for the three color genes involved. Segregating 1:1 for albino to nonalbino, 116 albinos tested gave an LD_{50} of 614.5 r and 119 nonalbinos gave a value of 650.2 r.

Although these data do not clearly differentiate between the two possible genetic sources of the albino sensitivity, they do indicate that the A strain is similar to the BALB strain in its contribution to this radiosensitivity. The test-cross albinos are made up of an A albino and a BALB albino gene, and the F_2 albinos are due only to the albino gene from the BALB parent. However, both albino groups show a comparable degree of radiosensitivity. Though this seems to give more weight to the albino locus specifically as the effective agent, the possibility that the determining genes are closely linked to this locus has not been eliminated.

Comparison of inbred and hybrid generations. The LD_{50} values and dosage-mortality slopes for the parent-inbred and hybrid generations are given in Table 33. The LD_{50} value of the F_1 generation is almost 30 r higher than the high parent and expresses a significant degree of hybrid vigor. The mid-parent LD_{50} , on the assumption of no dominance, would be 565 r, a value that is 95 r below the observed LD_{50} .

TABLE 33

Comparative data of parental strains, BALB/c and C57BL/6,
and their F_1 and F_2 hybrid generations

Strain	Observed $LD_{50}/30$	Expected* $LD_{50}/30$	Observed slope	Expected* slope
BALB/c	500.1 ± 6.9 r	-	$.368 \pm .032$	-
C57BL/6	630.3 ± 4.1 r	-	$.516 \pm .041$	-
F_1	659.5 ± 6.6 r	565 r	$.404 \pm .028$	.430
F_2	627.8 ± 6.6 r	548 r	$.274 \pm .026$	.261

*See text for derivation.

The F_2 LD_{50} falls back from the high value seen in the F_1 and approximates that of the high parent. If it is assumed that the F_2 mice should show a range of sensitivities encompassing the total range of both parent inbreds, then an expected slope and LD_{50} can be derived from a line joining the BALB $LD_{0.1}$ to the C57BL $LD_{99.9}$. The expected LD_{50} would be 548 r, a value that is 80 r below the observed. The deviation is not as great as that in the F_1 .

The dosage-mortality slopes present an altogether different picture from the LD_{50} values. Data on inbred strains⁽³⁾ gave rise to the hypothesis that the slope is as characteristic of the genotype as is the LD_{50} , and that the slope defines the innate individual variability in response. The slope of the F_1 is nearly intermediate in magnitude to the parent strains. Although the observed slope is not significantly different from the expected mid-parent slope, it is also not significantly different from that of the low parent. An assumption that the F_1 slope should be equal to the mid-parent value thus cannot be clearly established.

The F_2 slope, dropping sharply below those of the parent and F_1 generations, expresses the genetic variability of this segregating generation. The expected and observed slopes are exceptionally similar.

Thus, the slopes tend to express the genetic mixing to a high degree, if the assumption of no dominance is made. In contrast, the LD_{50} values manifest a significant amount of hybrid vigor. As a consequence, the high positive correlation between slope and LD_{50} noted previously in inbred strains⁽³⁾ does not hold up in the comparative analysis of inbreds with their hybrids. This does not invalidate the assumption that the slope is a valuable descriptive parameter of the genotype. Actually, the slope appears to be a parameter of even greater descriptive value than the LD_{50} with respect to transmission and segregation of the genetic factors controlling the acute lethal response to whole-body irradiation.

References

1. Grahn, D., and K. Hamilton. Acute lethal response of five inbred mouse strains to single dose total-body x-radiation. Quarterly Report of Biological and Medical Research Division, Argonne National Laboratory. ANL-5486, pp. 56-58 (1955).
2. R. J. Schweisthal and K. F. Hamilton. Effect of age, parity and litter size on the acute lethal response of four inbred mouse strains to whole-body x-irradiation. Quarterly Report of Biological and Medical Research Division, Argonne National Laboratory, ANL-5576, pp. 36-40 (1956).
3. _____, and K. Hamilton. Genetic variation in the acute lethal response of four inbred mouse strains to whole-body x-irradiation. To be published.

PROGRESS REPORT: DEUTERIUM OXIDE INTOXICATION IN RATS

Studies on Hypophysectomized Rats

John F. Thomson, Florence J. Klipfel, and Joseph J. Katz*

In a previous report,⁽¹⁾ data were presented on the effects of administration of heavy water to rats. These data showed that death occurred when the levels of D_2O in the plasma reached about 30%. The rats showed increases in blood urea, NPN, and inorganic phosphate, and decreases in glucose and plasma protein. Since a significant degree of adrenal hypertrophy was observed in these animals, we have repeated the experiment on hypophysectomized rats.

Methods

Hypophysectomized Sprague-Dawley rats were purchased from a commercial source. Their average body weight was 180 g. The rats were fed Arcady rat pellets supplemented by carrots and were given a 20% solution of sucrose in 50% D_2O as drinking water. Two experiments, each with 4 rats, were carried out. In the first one, 11 days elapsed between hypophysectomy and administration of D_2O ; in the second, the interval was 30 days.

Results

In the first experiment, the animals survived 6, 7, 9, and 12 days. Percentages of D_2O in the urine collected the day before death were, respectively, 13.0, 13.8, 24.1, not obtained. In the second series, the survival times were 6, 7, 7 and 13** days. The terminal concentrations of D_2O measured on either urine or plasma, were 17.4, 15.8, 16.0, and 22.4% D_2O , respectively.

Figure 30 shows the results of some analyses on blood and plasma of three of the four rats in the second series (the fourth rat died before any blood samples were taken). In the two rats which died on the seventh day, the NPN levels measured the day before showed increases to a degree seen in normal rats only at plasma levels of 28 to 30% D_2O .⁽¹⁾ Similarly, the decrease in glucose during the first 6 days was greater than that seen even in terminal samples of unoperated animals.

*From the Chemistry Division.

**This rat was obviously moribund, and was sacrificed after obtaining a terminal blood sample.

Figure 30

Changes in plasma protein, blood glucose, NPN, and urea in D_2O -treated, hypophysectomized rats. Rat X survived 6 days, rats $\circ$ and $\square$ 7 days, and rat Δ 13 days.

The failure of the third rat to respond as dramatically as the other two is not readily explainable. No evidence of residual pituitary tissue was seen at autopsy, there was no evidence of adrenal hypertrophy, and the animal did not gain weight at any time after operation. The only difference observed between this rat and the others in the series was that its water consumption was initially somewhat lower; it drank only 40 ml (20 ml of D_2O) during the first three days, while the other rats drank on the average 55 ml. During the next three days, however, this rat drank 70 ml, while the others continued to average 55 ml.

Changes in body weight were not marked. The average weight at death was 7 g less than the initial weight. There was no difference in weights of the adrenal glands between rats treated with D_2O and hypophysectomized controls at the same postoperative time interval.

Discussion

These data demonstrate that D_2O is much more toxic to hypophysectomized rats than to unoperated animals, primarily because the adaptation mediated in the intact rat by the adrenal cortex cannot occur after hypophysectomy.

In normal animals there was reason to believe that kidney damage was a principal cause of death.⁽¹⁾ These experiments indicate the possibility of a serious disturbance in carbohydrate metabolism, specifically gluconeogenesis, with emphasis on the liver as the organ most likely to be affected.

Reference

1. Thomson, J. F., F. J. Klipfel, and J. J. Katz. Deuterium oxide intoxication in rats. Quarterly Report of Biological and Medical Research Division, Argonne National Laboratory. ANL-5597, pp. 41-43 (1956).

PRODUCTS OF THE INDOLE-3-ACETIC ACID OXIDASE
FROM LUPINUS ALBUS L.

I. FORMATION OF INDOLE-3-CARBOXALDEHYDE

Robert E. Stutz

From the earliest investigations of the various indoleacetic acid (IAA) oxidases^(1,2) it has been presumed that indole-3-carboxaldehyde is the principal reaction product, although it could not be demonstrated experimentally. On the basis of chromatographic evidence⁽³⁾ it was thought to be formed by the bean root enzyme, and recently Racusen⁽⁴⁾ has reported similar evidence for the etiolated pea epicotyl enzyme. However, with the purified lupine enzyme, the amounts formed are too low to permit conclusive identification on the basis of chromatography of the enzyme reaction mixture.⁽⁵⁾

Since the oxidase and peroxidase activities in the lupine system could not be separated by starch electrophoresis between pH 5 to 9,⁽⁵⁾ an attempt was made to suppress the peroxidase action by removing protons via the hydrogen transport systems. The only methods found to be effective were to supply cytochrome C to the freshly prepared enzyme from etiolated lupine hypocotyls, or to supply cytochrome C, cytochrome oxidase, in the form of a rat liver homogenate, together with a phenol (e.g. 2,4-dichlorophenol) and Mn^{++} , to the purified enzyme.

The operation of the cytochrome system is accompanied by a decrease in oxygen uptake, the appearance of large amounts of indole-3-carboxaldehyde, and suppression of the formation of some of the other unknown reaction products, as is shown in an experiment in which the substrate was either carboxy- C^{14} -IAA or IAA-2- C^{14} (Figure 31). Only the #2 nonindolyl product was labeled in both cases; the labeled aldehyde was formed only from methylene-labeled IAA. Characterization of the aldehyde was carried out by comparison with the synthetic compound chromatographically, colorimetrically by the 2,4-dinitrophenylhydrazine and Ehrlich's tests, and by the ultraviolet and infrared spectrophotometric examination of fractions from the chromatograms.

The fraction soluble in ether at pH 10 was separated from 30 ml of the product mixture from the Warburg vessels and chromatographed, and the aldehyde area was rechromatographed several times. Chromatographic and ultraviolet spectrophotometric evidence was found for the formation of traces of the aldehyde in the absence of the cytochrome system.

Figure 31. Distribution of C^{14} activity from IAA- $2C^{14}$ among the reaction products is shown using a partially purified leaf enzyme preparation in 0.4 M tris buffer at pH 7.2. The flasks contained $3\mu M$ 2,4-dichlorophenol, $3\mu M$ Mn^{++} , 0.2 ml 5% rat liver homogenate, and $2 \times 10^{-5} M$ cytochrome C. Chromatography was carried out with an isopropanol:ammonia:water solvent on Whatman 3-mm paper; and the counts were taken on segments 1 cm in width. F is the solvent front, Ald is the indole-3-carboxaldehyde, #4 is an indolyl product minus its original carboxy group, #2 is nonindolyl but carries its original carboxy group, and the activity at the origin appears to be of a nonindolyl nature.

It is reasonable to assume that the aldehyde is formed via indole-3-glycolic acid, since it is formed from this substrate but not from indole-3-glyoxylic acid. Aldehyde formation appears to be dependent on the degree of removal of protons by the cytochrome system. The oxidation or peroxidation leading to nonindolyl products is thus diverted by the formation of the aldehyde. Identification of the other oxidation products is being pursued.

References

1. Tang, Y. W., and J. Bonner. The enzymatic inactivation of indoleacetic acid. I. Some characteristics of the enzyme contained in pea seedlings. Arch. Biochem. 13:11-25 (1947).
2. Wagenknecht, A. C., and R. H. Burris. The indoleacetic acid inactivating enzymes for bean roots and pea seedlings. Arch. Biochem. 25:30-53 (1949).
3. Stutz, R. E., and M. Eib. IAA inactivation. Report on Biological, Medical and Biophysics Programs, Argonne National Laboratory. ANL-5288, p. 63 (1954).
4. Racusen, D. Formation of indole-3-aldehyde by indoleacetic oxidase. Arch. Biochem. Biophys. 58:508-509 (1955).
5. Stutz, R. E. The indoleacetic acid oxidase from Lupinus albus L. Plant Physiol., in press.

MORTALITY OF CF No. 1 AND RF FEMALE MICE EXPOSED TO SINGLE DOSES OF Co^{60} GAMMA IRRADIATION

Howard H. Vogel, Jr.

When young adult female CF No. 1 mice were exposed to 90 min of whole-body irradiation with Co^{60} γ -rays in the Argonne gamma-neutron radiation chamber,⁽¹⁾ the $\text{LD}_{50/30}$ was determined to be 930 r.⁽²⁾ Several years ago Upton *et al.*⁽³⁾ exposed female RF mice at Oak Ridge National Laboratory to a Co^{60} source, and reported an $\text{LD}_{50/30}$ of approximately 750 r. It seemed improbable that this difference was due solely to the different mouse strains used in these experiments. Upton sent approximately 80 RF female mice from Oak Ridge to Argonne National Laboratory in order that the strain could be tested under the same experimental conditions in which our work with CF No. 1 had been carried out.

In a preliminary experiment, 56 RF mice were irradiated with a single dose of γ -rays (735-782 r). These exposures were carried out at a dose rate of approximately 16 r/min. The animals were exposed in three isodose groups: the group of 12 mice receiving the lowest dose (735 r) showed no mortality in 30 days; the remaining 42 mice showed approximately 10% mortality.

A second irradiation was then carried out, exposing 24 mice to 930 r with a resulting 62% mortality (see Figure 32).

Figure 32. Comparative 30-day mortality of RF and CF No. 1 female mice following single whole-body exposure to Co^{60} γ -rays.

It seemed clear from these experiments that the $LD_{50/30}$ for RF female mice exposed to Co^{60} in the gamma-neutron chamber at Argonne was approximately 900 r in comparison to 930 r reported for CF No. 1 females irradiated under the same conditions.

This experiment would seem to indicate that the rather large differences reported by the two groups would more probably be due to differences in dosimetry or in exposure facilities rather than to genetic differences in radiosensitivity between the two mouse strains. However, Upton has recently reported⁽⁴⁾ that there has been a marked increase in radiosensitivity in the RF female mice at Oak Ridge. Whereas males of this strain now respond to X-rays as they have for the past several years ($LD_{50/30} = 550$ r), the comparable figure for the females has increased by 36% to 750 r. The reason for the marked change in radiation sensitivity only among the females of the strain is not clear, but it certainly merits further investigation.

The Oak Ridge group has also reported that the γ -ray $LD_{50/30}$ for female RF mice taken from the colony at the same time that the mice were shipped to Argonne was approximately 1000 r (dose rate ~ 7 r/min), indicating again an unexplained shift in radiosensitivity among the females of this strain.

References

1. Vogel, H. H., Jr., R. Blomgren, and N. Bohlin. The gamma-neutron radiation chamber for radiobiological studies. *Nucleonics* 11 (No. 3): 28-31 (1953).
2. Clark, J. W., D. L. Jordan, and H. H. Vogel, Jr. Survival of CF #1 female mice after single acute exposures to Co^{60} γ -rays, to fast neutrons, and to mixtures of these ionizing radiations. *Radiation Research* 1: 128 (1954). (Abstract).
3. Upton, A. C., F. P. Conte, G. S. Hurst, W. A. Mills. The relative biological effectiveness of fast neutrons, X-rays, and γ -rays for acute lethality in mice. *Radiation Research* 4: 117-131 (1956).
4. ———, Personal communication (1956).

RADIATION RECOVERY. II. VARIATION OF THE TIME INTERVAL BETWEEN EQUAL PAIRED DOSES OF Co⁶⁰ GAMMA RADIATION

Howard H. Vogel, Jr., Verner Johnson*, Nicholas Bink, and Richard Barhorst

In a previous report⁽¹⁾ CF No. 1 female mice were exposed to paired equal doses of Co⁶⁰ γ -rays. The dose rate was constant at 16 r/min., but the interval between the two exposures was varied from 1 to 12 days. When this interval was only 24 hr, the LD_{50/30} was slightly but significantly increased (from 930 to 975 r).

The present experiment was carried out in order to decrease the interval between doses from days to minutes in order to study the recovery pattern under these conditions.

The irradiation was carried out in the high-level gamma room at a dose rate of 60 r/min. CF No. 1 female mice were randomly divided into 4 experimental and 1 control groups. Each group contained 32 animals (average weight: 25 g).

The experimental mice were placed inside individual plastic tubes and corks were inserted to prevent their escape. Eight of these tubes were then mounted on a standard masonite board which was placed at a known distance (23.5 in.) from the 1200-curie Co⁶⁰ source. The mice were all placed on shelf #3; the resulting mid-animal floor distance was 46 in.

The first experimental group was irradiated with 990 r delivered in 16.5 min. This same total dose was delivered to the other 3 experimental groups, except that the dose was divided into two approximately equal fractions (8.2 min and 8.3 min) with varying intervals between the doses.

Thus,

Group II received 492 r $\longrightarrow$ 10-min interval $\longrightarrow$ 498 r

Group III 492 r $\longrightarrow$ 30-min $\longrightarrow$ 498 r

Group IV 492 r $\longrightarrow$ 60-min $\longrightarrow$ 498 r.

The results of these four experiments are graphically portrayed in Figure 33, in which the per cent cumulative mortality is illustrated for each of the 4 experimental groups during the 30-day period following γ -irradiation. None of the control mice died during this period.

*Resident Research Associate from State College of Washington, Pullman, Washington.

Figure 33

Cumulative 30-day mortality of CF No. 1 female mice exposed to paired doses of Co^{60} γ -irradiation with intervals of 10 to 60 min between exposures.

Of the mice in group I, 28/32 (87.5%) died after exposure to a single whole-body dose of 990 r. Approximately a year ago, 191 CF No. 1 mice had been irradiated in this same gamma room with a single dose of 990 r delivered at 11 r/min.⁽²⁾ In this experiment, 167 (87.4%) died during the 30-day period after exposure. From these results a change of intensity from 11 r/min to 60 r/min does not seem to have any significant effect on 30-day mortality in this mouse.

It is evident from Figure 33 that there was recovery, as judged by cumulative mortality data, in each of the groups in which the dose was divided, and that intervals between γ -radiation of not more than 10 to 60 min. were sufficient to decrease mortality. It might be pointed out that $\text{LD}_{50/30}$ for this mouse has been reported as 930 r after single 90-min. exposure to Co^{60} .⁽³⁾ The results of group IV (1 hr between paired doses) were 15/32 dead or 47%. This would indicate that fractionating the dose with 1 hr between exposures reduced the mortality from 990 r to the equivalent of about 930 r or a dose reduction of ~60 r. Although this decrease in dose is less than 10%, it seems to be a real reduction.

It is interesting also that the amount of recovery is apparently related to the length of time between the paired doses, although there does not seem to be significantly greater recovery when this interval is increased from 1 hour to 1 day.

References

1. H. H. Vogel, Jr., J. W. Clark, and D. L. Jordan. Preliminary report: Recovery from Radiation I. The effect of paired equal doses of Co^{60} γ -rays on 30-day mortality in the mouse. Quarterly Report of Biological and Medical Research Division, Argonne National Laboratory. ANL-5597, pp. 65-69 (1956).
2. ———, J. W. Clark, and D. L. Jordan. The effect of dose rate of Co^{60} γ -radiation on 30-day mouse mortality, and its relationship to the "additivity" of fast neutrons and gamma rays. Quarterly Report of Biological and Medical Research Division, Argonne National Laboratory. ANL-5486, pp. 61-65 (1955).
3. Clark, J. W., D. L. Jordan, and H. H. Vogel, Jr. Survival of CF No. 1 female mice after single acute exposures to Co^{60} γ -rays, to fast neutrons, and to mixtures of these ionizing radiations. Radiation Research 1: 128 (1954). (Abstract)

RADIATION RECOVERY. III. THE RATE OF RECOVERY FROM RADIATION INJURY AS A FUNCTION OF AMOUNT OF INJURY

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

This preliminary experiment was designed to test the hypothesis that the rate (or rates) of recovery from radiation injury are a function of the amount of injury present. The general plan was to give varying doses of Co^{60} γ -rays to CF No. 1 female mice and then to determine, after a period of 5 days, the amount of additional radiation necessary to kill a portion of the animals in 30 days.

The five groups of 84 animals each are listed in Table 34. If one knows in each case the single dose which would result in the lethality observed after the two exposures (Column 5), it is necessary only to subtract the second dose (Column 3) to obtain a measure, in terms of dosage units, of residual injury from the first dose. This figure is shown in Column 6. The fraction of the original dose remaining at 5 days, and the recovery constants (day^{-1}), are also listed (Columns 7 and 8). The accuracy for Group I (which received for the first dose only 25% of the LD_{50}) is somewhat less than that for the remaining groups since the absolute value of the residual is small.

Figure 34 is a plot of the injury remaining after 5 days (Column 6), measured in r , as a function of the first dose. Figure 35 shows the relationship between the recovery constant and the injury, expressed as a fraction of the LD_{50} (single exposure). The recovery rate is probably at a maximum for doses less than half the LD_{50} .

TABLE 34

Residual dose and rate of recovery after γ -irradiation

Group*	First Dose		Second dose, r	Observed 30-day lethality	Estimated single dose to give observed lethality, r	First dose residual, Col. 5 minus Col. 3, r	Fraction of first dose remaining at 5 days	Recovery constant, day^{-1}
	r	Fraction of $\text{LD}_{50\ 30}$ (single exposure)						
	1	2	3	4	5	6	7	8
I	234	.25	836	.50	930	94	.40	.182
II	467	.50	738	.26	850	112	.24	.286
III	608	.65	650	.31	868	218	.36	.204
IV	776	.83	464	.62	967	503	.65	.087
V	884	.95	230	.75	1013	783	.89	.024

*Each group consisted of 84 mice.

Figure 34

Injury remaining after 5 days as a function of first dose.

Figure 35

Relation between daily recovery constant and injury, expressed as fraction of the LD_{50/30}, single exposure.

These data seem to indicate a progressive retardation of the recovery process as doses are increased above the level of half the single-exposure LD₅₀. One possibility is that the period of 5 days is sufficient for restitution of single chromosome breaks but not for appreciable replacement of injured cells.

In another report⁽¹⁾ we have compared the effects of variation in the time interval between equal paired doses of γ -rays, using the LD_{50/30} of mice as a criterion of recovery. When this time interval was 2 days the LD₅₀ was 1065 r, and for 6 days it was 1285 r. In the present series of experiments we did not use equal paired doses, but in one group receiving 605 r in the first dose and 663 r (after a 5-day interval), the mortality was 46%. This would seem to indicate that there is nothing really different about the 5-day interval between doses as some authors have recently reported.⁽²⁾

Lamarque and Gary-Bobo⁽³⁾ have reported very rapid recovery in the guinea pig following paired doses of γ -rays: Less than 25% of the first dose was residual when the interval between doses was only 24 hours. We believe this result can be explained by the high radio-sensitivity of the guinea pig, since one-half the LD₅₀ is less than 150 r and recovery after such a low first dose should be rapid.

Salerno⁽⁴⁾ recently reported that for doses less than 400 r given to CF No. 1 female mice, there was complete recovery after an interval of 12 days. In our experiments,⁽¹⁾ after a 12-day interval, 28% of the first dose was still "unrecovered." However, these results are not incompatible because of the absolute differences in the first doses: our experiments, 725 r; Salerno, less than 400 r.

References

1. Vogel, H. H., Jr., J. W. Clark, and D. L. Jordan. Radiation recovery. I. The effect of paired equal doses of Co⁶⁰ gamma rays on 30-day mortality in the mouse. Quarterly Report of Biological and Medical Research Division, Argonne National Laboratory. ANL-5597, pp.65-69 (1956).
2. Conte, F. P., G. S. Melville, M. Slater, and P. Ledford, Studies of cumulative injury from divided doses of X-rays and fast neutrons. Semiannual Progress Report of Biology Division, Oak Ridge National Laboratory. ORNL-1863, pp. 78-80 (1955).
3. Lamarque, P., and J. Gary-Bobo. A study of recovery following exposure of part or the whole of the body to ionizing radiation. Peaceful Uses of Atomic Energy. United Nations, New York (1956). Vol. 11, pp. 351-361.
4. Salerno, P. R. Rate of recovery of rats and mice exposed to X-rays. Fed. Proc. 15: 478-479 (1956).

MEAN SURVIVAL TIMES OF NEUTRON-IRRADIATED MICE

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

When CF No. 1 female mice were irradiated with fission neutrons (single, whole-body exposures for 90 min), the 30-day lethal range was found to lie between approximately 150 and 230 rads.⁽¹⁻³⁾ When mice were irradiated with less than 150 rads, no animals died during the 30-day period following exposure, whereas all mice irradiated with doses exceeding 230 rads died within this period.

If the dose was increased above this lethal range, from approximately 300 to at least 800 rads of fission neutrons, all the irradiated mice died on the fourth or fifth day following exposure. This broad dose range, resulting in mean survival times of approximately 4 days, seemed to correspond to Quastler's "intestinal death" in mice exposed to X-irradiation between ~ 1000 and $10,000$ r.⁽⁴⁾

It was of interest to determine whether mice irradiated with single doses in the region of $10,000$ rads would still show this 4-day death or if neurological symptoms would appear, indicating damage to the central nervous system. Therefore, 16 mice were irradiated with fission neutrons in the gamma-neutron chamber at CP-5 for a continuous 16-hour period at a neutron dose rate of approximately 9-10 rads/min.

Survival times in this experiment were calculated from mid-irradiation time. The first mouse died after 16 hr, 4 additional animals died between 30 and 40 hr, 9 died between 50 and 60 hr, and the last 2 between 80 and 85 hr following exposure. Thus the mean survival time of these 16 mice was approximately 52 hours from the mid-irradiation time (or 2.5 days from the beginning of the exposure).

None of the mice died in convulsions nor were any neurological symptoms evident. It seemed probable, however, that this $2 - 2\frac{1}{2}$ day death following exposure of mice to 10^4 rads of fission neutrons represented a different mortality mode from the 4-day intestinal mode. Further experiments will be carried out to substantiate this hypothesis.

Figure 36 illustrates the mean survival times (in days) of CF No. 1 female mice exposed to single whole-body irradiation with fission neutrons in the dose range $10^2 - 10^4$ rads.

Figure 36

Mean survival times of 30-day decedents, following single whole-body exposures of female CF No. 1 mice to fission neutron doses from $\sim 10^2$ to 10^4 rads.

References

1. Clark, J. W., H. H. Vogel, Jr., and D. L. Jordan. Progress report: Survival curves for CF #1 female mice after single 90-minute irradiation with fast neutrons and gamma rays (Co^{60}). Quarterly Report of Biological and Medical Research Division, Argonne National Laboratory. ANL-4948, pp. 32-38 (1953).
2. Vogel, H. H., Jr., J. W. Clark and D. L. Jordan. The daily probability of death in fast neutron and Co^{60} gamma-irradiated CF #1 female mice. Quarterly Report of Biological and Medical Research Division, Argonne National Laboratory. ANL-5378, pp. 118-120 (1955).
3. _____ J. W. Clark, and D. L. Jordan. Comparative mortality following single whole-body exposures of mice to fission neutrons and Co^{60} gamma rays. Radiology, in press.
4. Quastler, H. The nature of intestinal radiation death. Radiation Research 4: 303-320 (1956).

PELOMYXA PALUSTRIS FROM ILLINOIS

Edward W. Daniels

The giant multinucleated amoeba, Pelomyxa palustris Greef (1874), has been discovered in an abandoned quarry near Argonne National Laboratory. The site, which is known locally as Sag Quarry, is located some 25 miles southwest of Chicago, about 1/4 mile southeast of the highway #83 bridge over Sag Channel. The quarry is partly filled with water and the area is quite well developed ecologically. During the past year a dozen successful collections were made between October 11, 1955 and August 26, 1956 from a total of about 15 trips. Unusually rich cultures were obtained on November 16, 1955 and on May 20, 1956. Collections were made from bottom material about a yard from shore.

The natural habitat is characteristic for this species. The cytology of the nuclei, the number of nuclei, the cytoplasmic contents including food vacuoles (which possess plant material), and the large size of the cell also indicate clearly that this is P. palustris. Many specimens contained sand grains. The pseudopods formed by this organism, which are blunt and single or few in number, are characteristic of P. palustris.

This amoeba thrives on algae including diatoms and does not feed upon living protozoa or small metazoa as do Pelomyxa carolinensis and Pelomyxa illinoisensis. One living amoeba was observed under the microscope while it was in the process of taking in Spirogyra. The stick-like alga was drawn directly into the cell without elaborate, encircling pseudopodia. A small lip or ring-like pseudopodial ridge was formed at the entrance of the Spirogyra filament, and the filament fitted tightly into the vacuole as it entered. No definite antero-posterior orientation of the cell was made at this time.

Both living and stained, sectioned material were sent to Dr. R. R. Kudo who, since October of 1954, has been observing the organisms from a lake in New York.^(1,2,3) He agrees that this is P. palustris.

We have been able to keep it for some 3 months in the natural waters from which it was collected. While our original attempts at culture were unsuccessful, Kudo⁽²⁾ has recently indicated that laboratory culture is possible.

Laboratory culture of P. palustris organisms and a life cycle analysis will permit their extensive use as research animals. Some of the questions we propose to study, using this species, are the following:

1. Radiation sensitivity. Since there is a 10-fold difference in the LD₅₀ for X-rays between two other species of multinucleated giant amoebae, both of which are carnivorous,^(4,5) it would be of considerable interest to see what the radiosensitivity of P. palustris is in relation to these, particularly since its nutrition is vegetarian.

2. Cultivation in simple media. While none of the giant amoebae or Amoeba proteus⁽⁶⁾ have yet been obtained in bacteria-free culture, the observations of Holter and Marshall⁽⁷⁾ that P. carolinensis will take in protein through pinocytosis leads to the hope that this species of Pelomyxa may be adapted to cultivation on simple media.

3. Transplantation. Okada^(8,9) transplanted neutral-red-stained P. palustris over 25 years ago, but no further work was done. We propose to use this organism directly at a cellular and subcellular level as an aid to basic research on the problem of how grafts can be made to "take."

4. Radiation recovery problems. Andresen,^(10,11) Kudo,^(12,13,14) and Wilbur^(15,16) have contributed substantial cytological information on these amoebae as a group, and a considerable portion of the knowledge appears potentially useful in the study of radiation recovery problems. Intra-species or interspecies transplantation of various cell portions or particulates can be utilized in research directed toward the understanding of what is required by irradiated cells for recovery.

References

1. Kudo, R. R. Protozoology. 4th ed. Chas. C. Thomas, Springfield (1954).
2. ———. Personal communications (1955-1956).
3. ———. First Report, Institute of Microbiology, Rutgers Univ., N. J. pp. 52-53 (1954-1956).
4. Daniels, E. W. Studies on the effect of X-irradiation upon Pelomyxa carolinensis with special reference to nuclear division and plasmotomy. J. Exptl. Zool. 117:189-209 (1951).
5. ———. X-irradiation of the giant amoeba, Pelomyxa illinoisensis. J. Exptl. Zool. 130:183-197 (1955).
6. Prescott, D. M. Mass and clone culturing of Amoeba proteus and Chaos chaos. Compt. rend. trav. lab. Carlsberg. Sér. chim. 30: 1-12 (1956).

7. Holter, H., and J. M. Marshall, Jr. Studies on pinocytosis in the amoeba Chaos chaos. Compt. rend. trav. lab. Carlsberg. Sér. chim. 29:7-27 (1954).
8. Okada, Y. K. Transplantations versuche an Protozoen. Arch. Protistenk. 69:39-94 (1930).
9. ———. ["]Über den Bau und die Bewegungsweise von Pelomyxa. Arch. Protistenk. 70:131-154 (1930).
10. Andresen, Nils. Cytoplasmic components in the amoeba Chaos chaos Linne. Compt. rend. trav. lab. Carlsberg. Sér. chim. 24:139-184 (1942).
11. ———. Cytological investigations on the giant amoeba Chaos chaos L. Compt. rend. trav. lab. Carlsberg. Sér. chim. 29:435-555 (1956).
12. Kudo, R. R. Pelomyxa carolinensis Wilson. II. Nuclear division and plasmotomy. J. Morphol. 80:93-144 (1947).
13. ———. Pelomyxa carolinensis Wilson. III. Further observations on plasmotomy. J. Morphol. 85:163-176 (1949).
14. ———. Observations on Pelomyxa illinoisensis. J. Morphol., 88:145-184 (1951).
15. Wilber, C. G. The cytology of Pelomyxa carolinensis. Trans. Am. Microscop. Soc. 61:227-235 (1942).
16. ———. Origin and function of the protoplasmic constituents in Pelomyxa carolinensis. Biol. Bull. 88:207-219 (1945).

3-AMINO- 1,2,4-TRIAZOLE. I. EFFECTS ON RADIATION LETHALITY

Robert N. Feinstein and Sara Berliner

The title compound was first called to our attention by the work of Heim, Appleman, and Pyfrom,⁽¹⁾ who showed that after intraperitoneal injection of the agent into rats, the liver and kidney catalase activity was sharply reduced, while the catalase of the blood was unaffected. The first conclusion, of course, was tentatively to consider the amino triazole (AT) a catalase inhibitor; this appears improbable at present, as will be detailed in the immediately succeeding paper. Since such other catalase inhibitors as azide⁽²⁾ and cyanide⁽³⁾ have also been shown to have protective effects against the lethality of ionizing radiation,⁽⁴⁾ it was decided similarly to test AT.*

Table 35 indicates that AT injected intraperitoneally before whole-body X-irradiation rather consistently protects a large percentage of mice against 650 r and significantly prolongs the survival of animals receiving 750 or 850 r of X-ray. At higher doses of X-ray, the AT is without effect.

TABLE 35
Protection of mice by AT
against whole body X-radiation

Experiment	AT, mg/kg	X-ray, r	Order of treatment	No. of mice	% Survivors (30 days)	Av. sur- vival time, days†
1	0	450		10	90	9.0
	2000	450	AT, X-ray	12	92	13.0
	0	650		12	0	12.5
	2000	650	AT, X-ray	10	70	15.7
	0	850		12	0	8.9
	2000	850	AT, X-ray	12	8	10.6
	4000	0		11	100	-
2	0	850		8	0	6.3
	2250	850	AT, X-ray	12	8	9.7
	2250	0		12	100	-
3	0	850		11	0	11.5
	1817	850	AT, X-ray	11	0	13.5
	1817	850	X-ray, AT	11	0	11.0
	0	1700		11	0	4.5
	1817	1700	AT, X-ray	11	0	4.4
4	0	850		12	0	13.4
	1817	850	AT, X-ray	12	1*	13.3
	1817	850	X-ray, AT	12	0	12.1
	0	1700		11	0	4.0
	1817	1700	AT, X-ray	12	0	4.5
5	0	650		16	19	13.9
	2000	650	AT, X-ray	16	75	19.3
	0	750		16	0	11.1
	2000	750	AT, X-ray	13	0	13.6
	0	850		16	0	10.6
	2000	850	AT, X-ray	16	0	12.0
	2000	0		16	100	-

†Average survival time refers only to those animals succumbing within the 30-day period.

*We wish to thank Mr. T. M. Vial and the American Cyanamid Company for generous samples of technical grade 3-amino-1,2,4-triazole, and Dr. John H. Pomeroy and Mrs. Carolyn A. Craig for its purification.

Table 36 presents the results of a single instance (actually a composite of two experiments) where the interesting result was obtained that the combination AT, X-irradiation, and a second dose of AT, caused very rapid death of the animals, whereas the X-radiation alone caused the usual delayed death, and a double dose of AT, without radiation, had only slight toxicity. Attempts to duplicate these results have been negative, however, probably because such a dose of amino triazole is extremely close to the toxic level. Table 37 indicates that even if the AT is administered as long as 24 hours before the irradiation, some prolongation of survival time is conferred.

TABLE 36
Rapid death of mice
by combination of AT,
X-radiation, and repeated AT

Experiment	Treatment	No. of mice	% Survivors	Av. survival time, days*
1	X-ray alone	16	0	9.9
	AT, X-ray, AT	16	0	1.9
2	AT, X-ray, AT	14	14	2.6
	AT, AT	14	93	1.0

X-ray: 850 r

AT: 1817 mg/kg

*Average survival time refers only to those animals succumbing within 30 days.

TABLE 37
Effect of time lapse
between AT injection and
X-radiation on survival time

Time lapsed	No. of mice	% Survivors	Av. survival time, days*
5 min	12	8	9.7
30 min	11	0	10.9
1 hr	11	0	10.7
2 hr	12	0	9.5
6 hr	11	0	9.1
24 hr	12	0	8.4
No triazole	8	0	6.3

X-ray: 850 r

AT: 2250 mg/kg

*Average survival time refers only to those animals succumbing within 30 days.

100

References

1. Heim, W. G., D. Appleman, and H. T. Pyfrom. Production of catalase changes in animals with 3-amino-1,2,4-triazole. *Science* 122: 693-694 (1955).
2. Blaschko, H. The mechanism of catalase inhibitions. *Biochem. J.* 29: 2303-2312 (1935).
3. Rona, P., A. Fiegel and W. Nakahara. Liver and blood catalase. *Biochem. Z.* 160: 272-284 (1925).
4. Boyland, E. and E. Gallico. Catalase poisons in relation to changes in radiosensitivity. *Brit. J. Cancer* 6: 160-172 (1952).

3-AMINO-1,2,4-TRIAZOLE. II. EFFECTS ON TISSUE CATALASE: POSSIBLE SIMILARITY TO TUMOR GROWTH

Robert N. Feinstein, Sara Berliner, and Frank O. Green*

Heim *et al.* recently reported⁽¹⁾ that 3-amino-1,2,4-triazole (AT), upon intraperitoneal injection into rats, brought about a sharp reduction in the catalase activity of the liver and kidney, leaving blood catalase essentially unaffected. This anomaly, especially when coupled with our preliminary observation that AT is not itself an inhibitor of catalase activity, led us to investigate more thoroughly the relationship, both in vivo and in vitro, between AT and tissue catalase.

Experimental

All animal injections were intraperitoneal, at 0.01 ml/g of body weight. Concentrations used are given in each individual case. Aminotriazole used was a product of the American Cyanamid Co. and was used sometimes as the technical grade as received, and sometimes after recrystallization. Although significant differences in melting point and color were observed upon recrystallization, no difference was ever noted in the biological activity. Catalase activity was measured by the perborate method.⁽²⁾ Blood was assayed immediately; other tissues in in vivo experiments were lightly rinsed, quickly blotted, frozen in a dry ice-acetone bath, and stored in the deep freeze until assay.

Results

In vivo experiments. Table 38 indicates the effect of injected AT on the catalase of a variety of rat tissues. It is apparent that liver, kidney, and intestine are most sharply affected; testis, brain, and spleen somewhat less, and blood not at all. Because of the well-established⁽³⁾ fact that the presence of a tumor also reduces liver and kidney catalase, but not blood catalase, the Jensen sarcoma** was implanted into a group of six rats of the same shipment; when the tumor reached an adequate size (average 3.2% of total body weight), the animals were killed, and the corresponding tissues were assayed. It will be seen that the parallelism between the catalase activity of AT-injected and of tumor-bearing rats is not complete, contrary to the interesting suggestion of Heim *et al.*⁽¹⁾ that this might be so.

*Resident Research Associate from Wheaton College.

**We wish to thank Dr. Paul Talalay, University of Chicago, for the initial gift of a sarcoma-bearing rat, and Mrs. Mildred Summers for help with the first transplantations.

TABLE 38

Effect on tissue catalase of injection of AT and of presence of tumor

Tissue	Catalase activity				
	Perborate units per gram			% decrease from normal	
	Normal	AT-injected	Tumor-bearing	AT-injected	Tumor-bearing
Blood	210 $\pm$ 21	213 $\pm$ 15	183 $\pm$ 17	(-1)	13
Liver	1129 $\pm$ 165	72 $\pm$ 6	523 $\pm$ 146	94	54
Kidney	317 $\pm$ 33	25 $\pm$ 3	260 $\pm$ 19	92	18
Intestine	19.2 $\pm$ 1.2	2.4 $\pm$ 0.4	15.7 $\pm$ 2.0	88	18
Testis	10.0 $\pm$ 0.6	2.6 $\pm$ 0.2	8.7 $\pm$ 0.5	74	13
Brain	7.7 $\pm$ 0.6	5.0 $\pm$ 1.1	8.7 $\pm$ 0.5	35	(-13)
Spleen	81 $\pm$ 9	42 $\pm$ 9	75 $\pm$ 5	48	7

Data presented are the mean and the average deviation from the mean. Activity of blood samples is expressed as units per ml, rather than units per gram. Each datum is the average of the values for six rats. AT (1000 mg/kg) was injected intraperitoneally. The tumor was the Jensen sarcoma, implanted intramuscularly, and averaging 3.2% of the total body weight at time of sacrifice.

The data of Table 39 describe the effect of varying the AT dose and the time between injecting and sacrificing, on the catalase activity of the extirpated liver of the mouse. The speed with which the intraperitoneally injected AT reduces liver catalase activity is perhaps surprising. It is less surprising to see that with lower doses of AT, more time is required for inhibition to occur.

In vitro experiments. Since the catalase activity of many tissues is clearly reduced after intraperitoneal injection of AT, the obvious probability was that AT is an inhibitor of catalase. It was soon demonstrated that this was not the case. In repeated experiments, it was found that AT, mixed with mouse liver homogenate or with Armalase* (Armour's purified beef liver catalase) was without effect on the catalase activity if tested immediately after mixing or after 30 minutes incubation in the refrigerator. This was true with AT concentrations as high as 4.2% (0.5 M); if the AT concentration was increased to 8.4% (1.0 M), ** an inhibition of about 37%

*We wish to thank Mr. H. A. Orlove and the Armour Laboratories, Kankakee, Illinois, for a generous gift of Armalase.

**These values refer to the concentration of AT in the incubating mixtures; for assay, further dilution was ordinarily required.

TABLE 39

Effect of dose and time on reduction of
mouse liver catalase activity by AT

Experiment	Number of mice per datum	AT injected (mg/kg)	Time after injection	Catalase, P.U.*/g	% loss of activity
1	3	2250	15 min	42 ± 10	94
			30 min	35 ± 7	95
			1 hr	24 ± 5	97
			2 hr	22 ± 6	97
			6 hr	56 ± 5	92
			24 hr	243 ± 45	65
			(No AT)	686 ± 82	-
2	3	1690	1 min	878 ± 89	(-11)
			3 min	597 ± 28	24
			5 min	557 ± 41	29
			10 min	249 ± 28	68
			15 min	131 ± 16	83
			(No AT)	791 ± 33	-
3	4	85	10 min	735 ± 22	6
			15 min	612 ± 37	22
			(No AT)	785 ± 61	-
4	3	85	15 min	629 ± 60	23
			30 min	706 ± 63	14
			60 min	541 ± 33	34
			(No AT)	820 ± 36	-

*P.U. = perborate units. Data are mean and average deviation from the mean.

was detected even in the earliest possible assay. This, of course, would classify AT as an extremely mild catalase inhibitor. On the other hand, if the AT is incubated with liver homogenate for a period of time, a striking inhibition becomes apparent. This is apparently due to two phenomena: First, AT in high concentrations (higher than 0.5 M) apparently has some physical effect, presumably denaturation, on liver homogenates or on Armalase. This was shown by striking increases in the viscosity of liver-AT mixtures (after 4 hr incubation: relative viscosity in presence of 0.5 M AT, 4.1; in absence of AT, 1.6). It was shown also by the fact that fairly concentrated AT inhibits both liver homogenate catalase and Armalase after

incubation, while dilute AT inhibits liver homogenate catalase but not Armalase after incubation. Second, AT in lower concentrations in some as yet undefined way (presumably comparable to the in vivo effects) inhibits the catalase activity of liver homogenates after incubation. These statements are demonstrated by the data of Table 40.

TABLE 40

Effect of AT on catalase in vitro

Experiment	Catalase source	Conc. of AT, M*	Catalase activity at time shown P.U./ml					
			Initial	5 min	15 min	30 min	60 min	90 min
1	Mouse liver	0.00	1.691					
		0.01	1.671					
		1.00	1.031					
2	Mouse liver	0.00	1.216	1.214	1.218			
		0.01	1.240	1.018	0.914			
3	Mouse liver	0.00	1.313		1.351	1.357	1.352	1.411
		0.50	1.336		0.876	0.646	0.420	0.345
4	Mouse liver		Initial	2 hr	4 hr			
		0.00	1.700	1.859	1.625			
		0.02	1.671	0.740	0.017			
		0.10	1.667	0.713	0.007			
5	Mouse whole blood	0.00	1.720	0.214	0.007			
		0.50						
5	Mouse whole blood	0.00	0.931	0.929	0.933			
		0.25	0.895	0.237	0.074			
		0.00	1.718	1.735	1.772			
		0.02	1.742	1.748	1.750			
6	Armalase	0.10	1.719	1.642	1.604			
		0.50	1.720	1.464	1.429			

*Concentration of AT shown is that in the incubating mixture. For actual assay of catalase, it was often necessary to dilute this considerably further.

Concluding then that AT as such is not an effective inhibitor of catalase, the possibility appeared likely that the AT is converted, presumably enzymatically in liver homogenates, to some inhibitory substance. That such conversion, if it occurs, is probably enzymatic in nature seems likely from these considerations: (a) an active tissue (e.g., liver, whole blood) is necessary; blood serum will not serve; (b) incubation with Armalase causes no reduction of catalase activity (but, actually, rather a mild stabilization of activity in dilute Armalase solution); (c) AT incubated alone in aqueous solution does not become inhibitory; (d) AT incubated with liver homogenate in the refrigerator does not become inhibitory.

Attempts to demonstrate such an inhibitory substance have been uniformly negative. Early experiments were done with rather concentrated AT solutions and so must be repeated, since it is probable that under these circumstances catalase inhibition was brought about by denaturation; it is not necessary in these cases to postulate formation of an inhibitor, nor is it likely that enzymes needed to form such an inhibitor would be resistant to over-all denaturation. With properly diluted AT solutions, however, it has been possible to demonstrate that under circumstances in which sodium azide inhibition of catalase is largely released, AT inhibition is relieved much less or not at all. These dialysis experiments are presented in Table 41.

TABLE 41

Effect of dialysis on catalase activity of mouse liver homogenate inhibited with sodium azide or with AT

Experiment		Catalase activity, P. U./g liver		
		Homogenate diluted with:		
		H ₂ O	AT	Azide
1*	Initial	829	607	122
	After 2 hr at 37°, 2 hr at 0°C.	887	238	61
	After 2 hr at 37°, 2 hr dialysis at 0°C.	825	395	611
2**	Initial	715	722	
	After 19 hr at 37°, 4 hr at 0°C.	266	114	
	After 19 hr at 37°, 4 hr dialysis at 0°C.	195	79	

*Azide results limited by the dependence of catalase activity on the degree of dilution in the presence of azide. Azide or AT present at 0.02 M in incubating mixture.

**AT concentration: 0.02 M in incubating mixture.

Other considerations, discussed below, prompted the test of whether the hydroxy analog of AT, 3-hydroxy-1,2,4-triazole, was inhibitory. The compound was synthesized⁽⁴⁾ and was found to inhibit, at a concentration of 2×10^{-3} M, the activity of an Armalase preparation 21% after a 30-min period in the refrigerator. Under the same circumstances, the same concentration of AT actually "activated" the Armalase somewhat. (This is probably to be considered a stabilization against the expected deterioration of very dilute solutions of Armalase.)

One last test of mechanism should be noted here. It was found earlier⁽⁵⁾ that whereas the catalase of mouse liver was approximately 30% soluble and 70% bound to cellular debris, the catalase of guinea pig liver was approximately 80% soluble and only 20% bound to debris. Since another possibly important difference between the liver and the Armalase experiments with AT is the solubility or insolubility of the catalase, the effect of AT on guinea pig liver was tested. The AT was found to inhibit the guinea pig liver catalase approximately 65% under circumstances where mouse liver catalase would probably be inhibited well over 90%. This question will be further pursued.

In order to be quite certain that the results described above are in no way artifacts due somehow to the method of assay used, the effect of AT on liver homogenate was also determined by two other techniques. In the first, perborate was also used as substrate, but the incubation of enzyme and substrate was carried out for 30 seconds at 0°C ., instead of for 5 min at 37°C . In an earlier experiment⁽⁶⁾ it was shown that 5×10^{-4} M sodium azide inhibited liver catalase 40% by the usual technique of assay (incubation at 37° for 5 min) and 92% by the altered method (0° , 30 seconds). This modification permits the use of 1:200 liver homogenate instead of the usual 1:500 and so minimizes any possible dissociation of a catalase-inhibitor complex. Results were unchanged despite the change of techniques. In the second test, the "classical" determination of Katalase Fahigkeit (Kat.F.) was employed.⁽⁷⁾ The Kat. F.s of liver homogenate in presence and absence of 1×10^{-3} M AT were equal within 4%.

Discussion

Two interesting and fundamental questions remain unanswered. First, what is the mechanism of action of the aminotriazole on catalase, since it itself is neither an inhibitor nor, apparently, a precursor of an inhibitor? And second, why does it affect the catalase of most solid tissues but not that of blood, which has a fairly active catalase? The second question is also of interest to the cancer problem, for the same phenomenon occurs in the presence of a tumor. The first question, however, is of the most immediate interest, for its study may in turn answer the other.

It is certain that AT per se is not a catalase inhibitor of any potency. Even in concentrations which eventually will denature catalase, a catalase source is unaffected when freshly mixed with AT. This has been confirmed by the use of other techniques of assay. It is equally certain that AT does in some way strikingly affect tissue catalase activity; this is thoroughly documented by the data of Tables 39 and 40.

The enticing possibility that AT is converted to an inhibitory substance cannot be entirely ruled out but appears quite unlikely. All evidence thus far is purely negative, but this must probably be so. It is interesting that 3-hydroxy-1,2,4-triazole, which is a very plausible metabolic product of 3-amino-1,2,4-triazole (AT), is more inhibitory than AT, but even so this hydroxy analog must be considered a rather poor catalase inhibitor. Such inhibitors as sodium azide are better inhibitors by a factor of at least ten and, in addition, require no pre-assay incubation. It is, however, noteworthy that this is one more example of the generalization that aromatic hydroxides are more potent catalase inhibitors than the corresponding amines. Many more examples of this may be gleaned from the tables of Boyland and Gallico.⁽⁸⁾

Further evidence that it is not likely that an inhibitor is formed is provided by a later paper in this series,⁽⁹⁾ where it is shown that all AT injected into an animal, or mixed with a liver homogenate, can be accounted for as AT after a period of time.

Two other somewhat vague mechanisms remain to be scrutinized. One is the possibility that AT somehow interferes with the "turnover," or continuing renewal, of the catalase molecule. This appears rather unlikely if one considers the data of Table 39, where it is demonstrated that very sharp reductions in liver catalase are existent as soon as 10 min after intraperitoneal injection. The other possible mechanism in some way involves the structural integrity of the catalase source. Thus AT effect is sharpest in in vivo experiments (where presumably denaturation is not occurring), somewhat less noticeable in incubating mouse liver homogenate, still less apparent in guinea pig liver homogenate (where perhaps 80% of the catalase is soluble, in contrast to the 30% of the mouse liver homogenate), and not detectable at all in the case of totally soluble Armalase. Further experiments are planned to bear on this point.

References

1. Heim, W. G., D. Appleman, and H. T. Pyfrom. Production of catalase changes in animals with 3-amino-1,2,4-triazole. *Science* 122: 693-694 (1955).
2. Feinstein, Robert N. Perborate as substrate in a new assay of catalase. *J. Biol. Chem.* 180: 1197-1202 (1949).
3. Greenstein, Jesse P. *Biochemistry of Cancer*. Academic Press, New York (1954).
4. Green, F. O. 3-amino-1,2,4-triazole. V. Synthesis of the hydroxy analog. This report, p. 125.
5. Feinstein, Robert N., M. Hampton, and G. J. Cotter. Species specificity of catalase. *Enzymologia* 16: 219-225 (1953).
6. ———, and G. J. Cotter. Hydrogen peroxide as possible intermediate causative agent in radiation damage. XV. Mechanism of azide protection against ionizing radiation. U.S. Air Force Radiation Laboratory Quarterly Report 8: 77-79 (1953).
7. Sumner, J. B., and G. F. Somers. *Chemistry and Methods of Enzymes*. Academic Press, New York (1947).
8. Boyland, E., and E. Gallico. Catalase poisons in relation to changes in radiosensitivity. *Brit. J. Cancer* 6: 160-172 (1952).
9. Feinstein, Robert N., and F. O. Green. 3-amino-1,2,4-triazole. IV. Metabolism in mice. This report, p. 122.

3-AMINO-1, 2, 4-TRIAZOLE. III. QUANTITATIVE DETERMINATION OF THE COMPOUND

Frank O. Green* and Robert N. Feinstein

An analytical method for 3-amino-1, 2, 4-triazole (AT), to be employed in tracing the metabolism of the compound, should have certain desirable characteristics, particularly specificity, so that no interference will be caused by naturally occurring substances. In addition, of course, the method should have the usual characteristics desired in such a case: sensitivity, reproducibility, linearity with concentration, and simplicity. The method to be described satisfies all these requirements with the exception of that of linearity with concentration. Due presumably to a mildly interfering color which is constant and apparently an inherent consequence of the reagent employed, the curve of per cent transmittance vs concentration is not a straight line on semi-log paper, but rather a smooth curve. This deviation from Beer's law does not, however, prevent perfectly satisfactory reading of concentration values from the curve.

Experimental and Results

Principle. In principle, the method adopted consists of two parts: (a) the AT in trichloroacetic acid (TCA) solution is diazotized with nitrous acid, and (b) the diazo compound formed is coupled with 1, 8-dihydroxynaphthalene-3, 6-disulfonic acid (chromotropic acid, CTA) to form a colored derivative. TCA is the diazotizing acid of choice because it will simultaneously serve as deproteinizing agent to yield water-clear filtrates of most biological materials of interest. CTA was finally selected after investigating a variety of other possible coupling agents because it produces a pink color which absorbs well in the visible range, whereas most of the other agents tested gave light yellows which absorb well in the ultraviolet but poorly in the visible. A disadvantage of the CTA is that even in the absence of AT, it will itself produce a light yellow color. This color, however, absorbs rather poorly at 525 m μ , whereas the pink color absorbs maximally at this point. The interference is sufficient to cause deviations from Beer's law, but the curve of concentration vs. per cent transmittance, though curved on semi-log paper, is still usable for analysis. This curve is shown in Figure 37.

Procedure. The actual method consists of the following steps:

1. The solution being tested is mixed with an equal volume of 10% TCA, filtered if necessary, and the supernatant portion is used for analysis. If necessary to bring the AT concentration into the desired range, the filtrate is diluted with 5% TCA.

*Resident Research Associate from Wheaton College.

Figure 37. Concentration-absorption curve for aminotriazole analysis.

2. Five ml of the diluted filtrate is placed in a large test tube (ca. one-inch diameter). One ml of 0.01 M NaNO_2 solution is added and mixed by swirling.

3. One ml 0.0025 M CTA, made fresh daily, is added, the solutions are mixed by swirling, and the tube is placed in a boiling water bath for exactly 2-1/2 min and shaken intermittently.

4. The tube is placed in an ice bath for approximately 1 min, thus cooling it enough to minimize further reaction but not enough to cause dew formation in the spectrophotometer.

5. The solution is transferred to matched cuvettes and read at 525 $\text{m}\mu$, using as the standard a solution of 5% TCA which has been carried through the same procedure.

Stability of reagents. The only reagent requiring special attention is the chromotropic acid (CTA). This should be recrystallized before use and should be made up fresh daily from the purified crystals. Although a CTA solution could probably be used with impunity for several days, it does develop a more and more intense color with standing. This color, since it simultaneously occurs in the "blank" solution, does not affect the results significantly, but it seems wiser to prepare CTA fresh daily. The formation of the yellow color can be minimized by the inclusion in the CTA

solution of a small amount of ascorbic acid. However, for simplicity and because the CTA is so readily prepared fresh daily, it was decided to omit ascorbic acid or other stabilizer. Although solutions of sodium nitrite have not been shown to deteriorate significantly under ordinary conditions of storage, it is probably desirable to prepare this solution fresh weekly.

AT in 5% TCA has not been shown to deteriorate within a week or more. However, we ordinarily run analyses on the same day the samples are obtained.

Concentration range. With the instrument employed (the Coleman Junior Spectrophotometer), the usable range of concentration is approximately 4-50 μg of AT per ml of test solution, assuming a minimum of 3 ml of solution available. After addition of TCA and use of maximum (5 ml) aliquots, this means 10-125 μg total in the final solution.

Reproducibility. In four completely separate analyses with standard solutions, the average deviations at the extreme ends of the usable range were found to be approximately 2%.

Limitation in color development. The diazotization procedure is not critical in its timing, nor is it necessary to keep the solutions cold. The intensity of color developed during coupling, however, is critically dependent on time and temperature. If coupling takes place at room temperature, color development is completed after perhaps 2-1/2 hr. If color is developed at 37° C, the color development is essentially complete within an hour, but the yellow color of the blank is still increasing; although, as stated earlier, this interferes very little, there seems little advantage to the use of a 37° bath. If color is developed in a bath of boiling water, maximum color appears after about 2-1/2 min heating; after this, some bleaching occurs. The extent of this effect is shown in Table 42.

TABLE 42

Effect of heating time in coupling AT
with chromotropic acid

Time in boiling water bath, min	Optical density	
	AT solution	"Blank" solution
1.0	.508	.141
1.5	.548	.156
2.0	.551	.161
2.5	.555	.161
3.0	.546	.150
3.5	.542	.159
4.0	.542	.159
4.5	.530	.153

Specificity. The method presumably determines only aromatic amines. The only individual compound thus far tested is the hydroxy analog of AT, 3-hydroxy-1,2,4-triazole. Approximately 130 μ g of this compound failed to produce any detectable coloration. To demonstrate the absence of chromogenic substances in biological material customarily used, a whole mouse was disintegrated in the Waring blender and filtered through gauze. Proteins were precipitated as usual, and the supernatant analyzed as described. Approximately 15 μ g of AT-equivalent chromogen was detected in the entire carcass.

3-AMINO-1,2,4-TRIAZOLE IV. METABOLISM IN MICE

Robert N. Feinstein and Frank O. Green*

In a previous paper of this series, ⁽¹⁾ this question was raised but not settled: How can 3-amino-1,2,4-triazole (AT) so strongly inhibit tissue catalase after intraperitoneal injection when it is not itself a catalase inhibitor nor, apparently, converted to a catalase inhibitor? When an analytical method for this compound became available, ⁽²⁾ other possible lines of attack were opened. The present paper will discuss some preliminary experiments bearing on the metabolism of the compound, both in vivo and in vitro.

Experimental and Results

AT assays were performed according to the technique described in a previous paper. ⁽²⁾ Mice were injected intraperitoneally with doses of 845 mg AT per kg body weight. When the AT content of an entire mouse was to be determined, this was done as follows: The mouse was killed by cervical dislocation, dropped into a Waring blender with nine times its own weight of water, blended 1 min, and filtered through gauze. Aliquots of the filtrate were treated with an equal volume of 10% trichloroacetic acid (TCA) to precipitate proteins. The mixture was centrifuged, and the supernatant was assayed for AT, diluting first with 5% TCA to bring the concentration within the desired range. In in vitro experiments, e.g., liver homogenate treated with AT, the reaction between the liver and the AT was halted at the desired time by the addition of an equal volume of 10% TCA, and the AT was assayed as described above.

In the first experiment, mouse liver homogenate was mixed with an equal volume of 0.004 M AT, and the mixture was assayed for AT immediately and at hourly intervals for 5 hr at 37° C. Under these circumstances, it would be expected that catalase activity of the liver homogenate would have decreased by 99% within 4 hr. However, within the limits of experimental accuracy, 100% of the original AT could be accounted for as AT at the end of 5 hr.

Next, the possible in vivo metabolism of the AT was measured. Nine mice were injected intraperitoneally with 845 mg of AT per kg body weight. Three were sacrificed immediately, 3 at 3 hr, and 3 at 6 hr. The whole animals were separately analyzed for AT as described above. During the 3- or 6-hr waiting periods, the mice were individually housed in beakers, and at the time of sacrifice the beakers were thoroughly rinsed out with the water to be used for blending. Thus the assay included mouse and excreta. Again, within experimental error all injected AT was accounted for as AT. Actual data are provided in Table 43.

*Resident Research Associate from Wheaton College.

TABLE 43

Recovery of AT from mice after intraperitoneal injection*

Time after injection	Dose AT injected, mg	AT recovered, mg	% AT recovered	Wt. of mouse, g
Zero	24.5	27.8	113	29
	22.0	23.7	108	26
	22.8	24.3	107	27
			Av. 109%	
3 hr	20.3	17.8	88	24
	23.7	23.8	100	28
	22.8	27.0	118	27
			Av. 102%	
6 hr	22.8	25.9	114	27
	22.8	25.9	114	27
	22.0	28.3	129	26
			Av. 119%	

*Recovery was from combined carcass and excreta. See text.

To determine whether the AT remained in the body of the animal or was excreted, three mice were injected with 845 mg of AT per kg, housed in ordinary cages for 24 hr, then sacrificed and assayed individually for AT. In this case excreta were not retained. No trace of AT could be detected in the carcass of any animal.

Although the preceding two experiments jointly indicate that all injected AT is excreted unchanged, the conclusive proof would be the demonstration of all injected AT in the excreta.

Results in this direction are not completely satisfactory as yet, and the experiments are being repeated and amplified. However, the following conclusions seem fairly well established:

(a) The great bulk of AT injected, either intraperitoneally or intravenously, is excreted in the urine. With a single exception, all experiments (six now bear on this point) indicate a urinary recovery of 85% to over 100% of the injected AT. The percentage recovery, with the single exception noted, is not dose-dependent, indicating that deviations are more likely due to analytical variation than to metabolism of AT.

(b) Some AT is excreted in the feces. However, this route of excretion is quite minor and accounts for only 2-3% of the total injected AT.

Discussion

The possibility cannot yet be excluded that the AT is changed, upon injection or upon incubation with liver homogenate, to a substance which is not only a catalase inhibitor but which reacts, both qualitatively and quantitatively, like AT in the analytical color reaction employed. This seems highly unlikely; however, from considerations of the color reaction used. The reactions require, presumably, an amino group attached to an aromatic ring. Thus, removal of the 3-amino side chain would cause loss of the reaction. In fact, it has been demonstrated⁽²⁾ that the 3-hydroxy analog gives no color in this reaction. Further, almost any change in the ring, whether cleavage or saturation, will reduce the aromaticity of the ring and, presumably, cause loss of its ability to diazotize and couple. Thus the most plausible metabolic pathways of the AT seem unlikely. The remaining possible pathway, that of some sort of conjugation such as is typical of many detoxification mechanisms, cannot yet be excluded.

References

1. Feinstein, R. N., S. Berliner, and F. O. Green. 3-amino-1,2,4-triazole. II. Effects on tissue catalase. Possible similarity to presence of tumor. This report, p. 110.
2. Green, F. O., and R. N. Feinstein. 3-amino-1,2,4-triazole. III. Quantitative determination of the compound. This report p. 118.

3-AMINO-1,2,4-TRIAZOLE V. SYNTHESIS OF THE HYDROXY ANALOG

Frank O. Green*

The first attempt at the synthesis of 3-hydroxy-1,2,4-triazole (HT) used the common method of warming the diazonium derivative of 3-amino-1,2,4-triazole (AT) in water to replace the amino with the hydroxyl group. The proposed reactions were as follows:

This apparently straightforward method, however, failed to produce anything resembling the desired product. A search of the literature revealed⁽¹⁾ that although the triazoles generally may be satisfactorily diazotized and coupled, warming the diazonium compound in water will ordinarily replace the amino group with the anion of the acid present. Thus in the present case, warming the diazotized AT in the presence of HCl produced white crystals melting at 167-169° C. (mp of 3-chloro-1,2,4-triazole: 167°C.)⁽¹⁾ Warming the diazotized AT in the presence of HNO₃ produced an explosive compound which, though not further investigated, was presumably 3-nitro-1,2,4-triazole.

The second attempt at synthesis of the HT was based on the report of Widman and Cleve.⁽²⁾ The reaction is between acetone semicarbazone and formic acid and presumably would be formulated as follows:

Literature directions for this synthesis are incomplete, particularly as regards quantities of reagents. Some experimentation was therefore required, and it was found that the particular product obtained was critically dependent on the ratio of formic acid to acetone semicarbazone. Using a molar ratio of 4:1, there was obtained a white compound melting sharply at 235.5 - 236.5°C. The compound was analyzed as follows:

*Resident Research Associate from Wheaton College.

<u>Element</u>	<u>Calculated</u>	<u>Found</u>
C	28.24%	28.31%
H	3.55	3.70
N	49.40	50.10

Discrepancies between this compound and those in the literature description⁽²⁾ lie in the fact that the presently synthesized compound is white, while the other was described as lightly yellow; that the present compound is not very soluble in absolute alcohol, while the other was described as very soluble in this agent; and that the older melting point was stated to be 234°, against the presently determined 236°.

References

1. Morgan, G. T., and J. Reilly. Non-aromatic diazonium salts. V. Diazo-derivatives of aminotriazole. J. Chem. Soc. (Trans., Part 1) 109: 155-160 (1916).
2. Widman, O., and A. Cleve. Ueber das 3-Oxy-1,2,4-triazol und einige Acidylsemicarbazide. Ber. 31: 378-381 (1898).

PROGRESS REPORT: STUDIES WITH A CHLOROLEUKEMIA IN THE RAT

Harvey M. Patt, Eugenia M. Jackson, and Mary A. Maloney

The growth characteristics of a myelogenous chloroleukemia in the rat are being investigated as part of a general program on mechanisms of leucocyte regulation. The tumor originated in a rat of the Sprague-Dawley strain about one year after injection of actinium.* It has been maintained since by serial transfer of blood or chloroma from tumor-bearing rats to infant animals. The objectives of the present experiments are: (a) to determine the dosage requirements for transfer by blood, marrow and chloroma, (b) to evaluate the growth response as a function of route of inoculation, age, and sex, and (c) to ascertain on the basis of the above whether it is possible to dissociate the leukemia from the chloroma.

Blood and chloroma were obtained 3 to 4 weeks after intraperitoneal inoculation of a tumor suspension into several-day-old Sprague-Dawley rats. There was a well-developed tumor mass in the peritoneal cavity and a beginning leukemoid blood picture at the time of sacrifice. The chloroma was minced, suspended in saline solution and filtered through a fine wire mesh. Varying volumes of blood or chloroma suspension were injected intraperitoneally into 2- to 7-day-old rats. The mortality data are presented in Table 44. Blood counts and body weights were also obtained in some animals and will be reported later. It will be noted that 50% of the animals receiving 0.2 ml of blood and 90% of those inoculated with 0.5 ml were dead within 3 days. In contrast, there was only a small early attrition in the 0.05 ml blood group and in the 3 chloroma groups. This is perhaps attributable to the trauma incidental to injection. The acute killing in the intermediate and high blood-inoculum groups, which could be explained in several ways, requires further study. The subsequent mortality pattern is similar in the low and intermediate blood groups. There is, however, the suggestion of a graded lethal response in the infant rats receiving varying amounts of the chloroma suspension. The chloroma-injected animals died sooner than animals inoculated with blood. Although the latter received considerably fewer cells (excluding erythrocytes), it is not possible at this time to make a quantitative comparison in terms of the cell types that may be critical for transplantation.

Transfer of the chloroleukemia to newborn recipients is not a requirement since takes have been achieved in 7- to 8-week-old rats. The results summarized in Table 45 reveal that the mortality rate in the older animals is greater with intraperitoneal than with subcutaneous injection. There is also the suggestion that tumor development with either route of administration may be somewhat more rapid in males than in females.

*Mound Laboratory; the tumor was obtained through the courtesy of Marietta Miller.

TABLE 44

Survival of new born rats inoculated with a chloroleukemia

Transfer group	Volume injected, ml	Number of animals	% Mortality											
			Time after inoculation, weeks											
			3 days	1	2	3	4	5	6	7	8	9	10	
Blood (26×10^6 leucocytes/ml)	0.05	30	10	13	17	17	17	17	33	77	77	83	90	
	0.20	33	48	48	48	48	48	48	61	88	91	97	97	
	0.50	24	92	92	92	92	92	92	92	96	96	96	96	
Chloroma (222×10^6 cells/ml)	0.05	23	13	17	17	22	35	78	100					
	0.20	20	10	10	15	30	45	90	100					
	0.50	27	19	19	19	44	63	100						

TABLE 45

Survival of 7- to 8-week-old rats inoculated with a chloroleukemia
(2.0 ml of a suspension containing 222×10^6 cells/ml)

Group	Number of animals	% Mortality								
		Time after inoculation, weeks								
		4	5	6	7	8	9	10	11	
Male I.P.	17	0	29	71	94	94	94	94	94	
Male subcutaneous	16	0	0	19	19	25	25	44	56	
Female I.P.	21	0	0	24	76	86	90	90	95	
Female subcutaneous	21	0	0	0	5	19	19	29	38	

PROGRESS REPORT: FORMATION CONSTANTS FOR THE COMPLEXES OF COBALT AND OF ZINC IONS WITH CITRIC AND WITH OXALIC ACID

Edmund L. Lind*

Formation constants have been determined for the complexes of cobalt and zinc with citric and oxalic acids between pH values of 0.8 and 7.25. The ion exchange technique was used; all solutions were at an ionic strength of 0.16; sodium chloride was the bulk electrolyte except at pH 0.8, in which case 0.16 M perchloric acid was used. Substantially carrier-free Co^{60} and Zn^{65} were used as the metallic ions.

Formation constants determined at pH values in the neighborhood of 7.25 correspond to complex formation between metal ion and citrate ion (Cit^{3-}), or oxalate ion (Ox^{2-}), in the cases of the ligands citric acid and oxalic acid, respectively. At pH 0.8, the complexing species appear to be mainly H_2Cit^- and HOx^- , although this conclusion is a tentative one so far as complete exclusion of complexing by the unionized ligands is concerned. Values for the HCit^{2-} complexes were obtained from measurements at intermediate pH values.

The following tentative $\text{Log } K_f$ values have been assigned:

	<u>Co</u>	<u>Zn</u>
Cit^{3-}	4.6	4.7
HCit^{2-}	3	3
H_2Cit^-	1	1
Ox^{2-}	3.8	3.8
HOx^-	(to be calculated)	

*Resident Research Associate from Central Washington College of Education, Ellensburg, Washington.

PROGRESS REPORT: RADIOELEMENT REMOVAL

III. Manganese Removal

Joan F. Fried, Marcia White Rosenthal, and Elizabeth Moretti

In a previous report⁽¹⁾ we described a new chelating agent with low Ca affinity, namely Chel 138, which is being investigated in our laboratory for effectiveness in removal of metals from the body. Titration studies indicated that this compound, which is an analog of ethylenediaminetetraacetic acid (EDTA)⁽²⁾ binds Mn in vitro more effectively than EDTA. Since calcium EDTA has been reported to be of therapeutic value in the removal of Mn from livers and brains of rats poisoned with this metal⁽³⁾ it seemed advisable to study the effect of Chel 138 on the distribution of Mn in the tissues and excreta of rats given trace amounts of Mn⁵⁴, and on survival of rats given an LD₉₀ of Mn gluconate (stable isotope).

Preliminary Toxicity Study

A preliminary toxicity study was conducted on Chel 138 using 13-month-old Sprague-Dawley female rats. A solution of Chel 138 was prepared as the tetrasodium salt and determined by solids analysis to be 2.1% concentration. The experimental plan and results are shown in Table 46.

From this data it was estimated that the 30-day LD₅₀ for a single intravenous injection would be 100-120 mg/kg; this compares with 100 mg/kg for Versene (the tetrasodium salt of EDTA).⁽⁴⁾ A safe therapeutic dose for repeated daily injections was estimated to be 80 mg/kg for the first day followed by 40 mg/kg for subsequent days.

Gross autopsy findings were consistent in showing a picture of liver congestion, kidney pathology together with deposition of what appeared to be solid particles of the Chel 138 in the cortex of the kidney, some caecal hemorrhages and general dilation of all large gastrointestinal blood vessels.

Distribution Study

Materials. Sixteen 5-month-old Sprague-Dawley female rats weighing 250-280 g were used. Chel 138 was prepared in 2.1% solution as the tetrasodium salt and the pH was adjusted to 7.2. Ca EDTA was prepared in 2.05% and in 6.15% solution as calcium disodium EDTA. The Mn⁵⁴Cl₂ injection solution, kindly prepared by W. Kisielecki, contained ~ 2 μ c Mn⁵⁴ per ml in isotonic saline (carrier-free solution).

TABLE 46

Toxicity of Chel 138 in rats

Daily injection, ml	Route	No. days injected	No. rats	Daily dose, mg/kg	Total dose, mg/kg	No. deaths	30-day survival, %
4.0	I.P.	2	2	284	568	2	0
3.0	I.P.	2	2	214	428	2	0
2.0	I.P.	1	2	140	140	2	0
1.5	I.V.	2	1	103	206	1	0
1.5*	I.V.	3	1	109	254	1	0
1.0**				72.5			
1.0	I.V.	3	3	69	207	0	100
0.75	I.V.	3	2	53	159	1†	50
0.50	I.V.	3	3	34	102	0	100
0.35	I.V.	3	3	22	66	0	100

*1.5 ml injection on first day.

**1.0 ml injection on second and third day.

† Dead after second injection.

Methods. The experimental plan is shown in Table 47. Animals in groups 1, 2, 3 and 4 were placed in metabolism cages, and urine and feces were collected separately for each 24-hr period from time of injection to time of sacrifice. At time of sacrifice the following tissues were removed from all animals and weighed: pancreas (whole), liver (whole), kidneys (whole), brain (longitudinal half), and femurs (whole). The soft tissues (except the liver) and the femurs were placed in lusteroid tubes containing 4 ml 3 N HCl. The femurs were pressed to the bottom with a glass rod after 2 days during which decalcification had become nearly complete. Livers were ashed in beakers with concentrated HNO₃ and dried. The ash was digested with concentrated H₂SO₄ until the solution became clear and colorless. The solution was then diluted to 50 ml volume with 3 N HCl from which a 3-ml aliquot was removed and placed in a glass counting tube. Standard tissues were prepared in all cases by adding appropriate volumes from a "dummy bottle" containing 2% of the injected dose per ml to tissues from the control animal (group 7). Urine and feces were diluted to 100 ml with 3 N HCl. Standard urine and feces samples were prepared by addition of "dummy" solution to collections from the animal in group 7. A standard carcass was taken from the animal in group 6.

TABLE 47

Experimental plan for distribution study

Group	No. rats	Mn ⁵⁴ Cl ₂ I.V., μc	Treatment I.V.			Time of sacrifice after Mn ⁵⁴ injection
			Time after Mn ⁵⁴ injection			
			1 hr	2 days	3 days	
1	3	1	Chel 138, 80 mg/kg	Chel 138, 40 mg/kg	Chel 138, 40 mg/kg	4 days
2	3	1	Ca EDTA*	Ca EDTA*	Ca EDTA*	4 days
3	3	1	Ca EDTA, 200 mg/kg	Ca EDTA, 200 mg/kg	Ca EDTA, 200 mg/kg	4 days
4	3	1	Saline	Saline	Saline	4 days
5	3	1	None	--	--	1 hr
6	1	1	--	--	--	Immediate
7	1	0	None	None	None	4 days

*Equimolar to Chel 138 in Group 1.

Tissues were counted in a NaI(Tl) well counter in conjunction with a single-channel, pulse height analyzer at the optimum window setting for the 0.84-MEV γ -ray emitted by Mn⁵⁴ (channel width 450, baseline 75). Feces and urine were counted in volumetric flasks which were placed in a pressurized ionization chamber. This instrument is designed to permit 100% counting geometry and is sensitive to 0.06 μ c Mn⁵⁴. Carcasses were also counted in this manner.

Calculations were made as follows:

$$\frac{\text{count per sample}}{\text{count per standard}} \times (\% \text{ injected dose in standard}) = \% \text{ injected dose per sample}$$

Standard deviation was calculated according to the formula

$$\text{S.D.} = \sqrt{\frac{\sum (v - \bar{v})^2}{N}}, \quad \text{where } v = \text{variable, } \bar{v} = \text{mean value of variables, and}$$

N = number of samples.

TABLE 48
Total Distribution of Injected Dose
(% injected dose per whole organ)

Group	Therapy	Rat	Time of sacrifice	Both femurs	Carcass	Brain	Pancreas	Both kidneys	Liver	Urine	Feces	Blood	Total
1	Chel 138, 80 mg/kg	1	4 days	0.23	20.3	T.L.T.C.*	2.35	1.78	14.40	T.L.T.C.	63.26	- †	102.3
		2	4 days	0.25	23.4	T.L.T.C.*	1.92	2.51	15.95	T.L.T.C.	73.55	-	117.6
		3	4 days	0.27	21.6	T.L.T.C.*	2.63	2.55	16.22	T.L.T.C.	73.48	-	116.2
		Av.		0.25 ± .02	21.8 ± 1.3		2.30 ± 0.27	2.28 ± 0.35	15.52 ± 0.80		70.10 ± 4.83		
2	EDTA, equimolar	4	4 days	0.25	20.7	T.L.T.C.*	3.07	1.67	17.65	7.13	59.33	-	109.8
		5	4 days	0.24	21.4	T.L.T.C.*	2.75	1.82	17.72	5.67	58.97	-	108.6
		6	4 days	0.17	21.2	T.L.T.C.*	2.85	1.65	16.55	4.82	60.58	-	107.8
		Av.		0.22 ± .04	21.1 ± 0.3		2.89 ± 0.13	1.71 ± 0.08	17.31 ± 0.97	5.87 ± 0.96	59.63 ± 0.79		
3	EDTA, 200 mg/kg	10	4 days	0.22	22.3	T.L.T.C.*	2.36	1.17	14.14	15.07**	45.87**	-	101.2
		11	4 days	0.21	21.2	T.L.T.C.*	2.09	1.50	16.30	15.07**	45.87**	-	102.2
		12	4 days	0.17	21.5	T.L.T.C.*	2.44	1.54	15.87	15.44	46.02	-	103.0
		Av.		0.20 ± .03	21.7 ± 0.5		2.30 ± 0.15	1.40 ± 0.17	15.44 ± 0.93	15.20 ± 0.18	45.95 ± 0.07		
4	Saline	13	4 days	0.20	28.3	T.L.T.C.*	2.70	2.20	13.40	0.92	55.19	-	102.9
		14	4 days	0.23	23.5	T.L.T.C.*	2.99	2.13	15.70	T.L.T.C.	59.70	-	104.3
		15	4 days	0.19	20.4	T.L.T.C.*	2.65	1.97	14.59	T.L.T.C.	70.17	-	110.0
		Av.		0.21 ± .02	24.1 ± 3.2		2.78 ± 0.15	2.10 ± 0.20	14.56 ± 0.94		61.69 ± 6.28		
5	None	16	1 hr	0.38	27.4	< 0.05	2.08	3.30	71.87	-	-	T.L.T.C.	105.0
		17	1 hr	0.36	33.3	< 0.05	1.81	2.93	58.90	-	-	T.L.T.C.	97.3
		18	1 hr	0.36	30.8	< 0.05	1.88	3.34	61.41	-	-	T.L.T.C.	97.8
		Av.		0.37 ± .01	30.5 ± 2.0		1.92 ± 0.13	3.19 ± 0.18	64.06 ± 5.60				

*T.L.T.C. = Too low to count -- less than 2 x background.

**The values for animals No. 10-11 represent ½ of the combined urinary or fecal excretion for the two since these animals were caged together in one metabolism cage.

† Dashes indicate that no samples were taken.

Results. No significant differences are present in the distribution of Mn among the tissues of the Chel 138 treated animals as compared with the saline controls (Table 48). The route and extent of Mn excretion were also unaltered by the Chel treatment. An equimolar dose of Ca EDTA promoted a small but significant increase in excretion of Mn in the urine with what appears to be a corresponding decrease in the feces. Ca EDTA at a larger dose (200 mg/kg) had a greater effect on this shift of excretion from the feces to the urine. Ca EDTA at both doses promoted a decrease in kidney content of Mn which may possibly be related to the increase in urinary excretion. In connection with this, it is interesting that the kidneys of 2 of the 3 animals treated with Chel 138 were observed at time of sacrifice to contain tan deposits in the outer cortex which may have been particles of solid Chel 138 or Chel-Mn complex; Chel 138 is known to be less soluble at the pH of the kidney. Neither Chel 138 or Ca EDTA had any effect on the quantity of Mn in the liver or on total retention of injected dose.

Discussion. The results obtained with Ca EDTA treatment agree with those of Maynard and Fink⁽⁵⁾ who injected Mn as the EDTA chelate and observed increased urinary and decreased fecal excretion of the Mn in the chelated form. The data from animals sacrificed at time of treatment shows that the greater percentage of the injected dose is found in the liver at 1 hr. This is in agreement with findings of others⁽⁶⁾ who have also shown that about 40% of the manganese retained in the liver is incorporated into the mitochondria, and about 30% is distributed equally between the microsomes and nuclei of the liver cells. The failure of either EDTA or Chel 138 to bring about increased total excretion by means of a reduction of liver content is probably explained by the inability of these two agents to penetrate the cell membranes. It will be seen (Table 48) that the blood level of Mn is very low at the time of injection of the chelating agents, and therefore there was little or no contact between these agents and circulating Mn⁵⁴.

Survival Study

The experiment described above showed no significant effect of Chel 138 administration on distribution or excretion of injected manganese in tracer quantities. However, the possibility remained that Chel 138 could prove therapeutically effective against an LD₉₀ of manganese through an in situ inactivation mechanism, similar to the action of ATA against beryllium. A survival experiment was therefore conducted.

Twenty Sprague-Dawley female rats, aged 9 weeks, were given manganese gluconate (25 mg/kg) intraperitoneally. One and one-quarter hours later Chel 138 (60 mg/kg) was administered intravenously to 10 of these; the other 10 animals received no treatment. The group treated with Chel 138 showed a 20% survival whereas the uninjected control group showed a 10% survival; all deaths in both groups occurred in less than 24 hr. The difference in survival is not significant.

On the basis of these two experiments the conclusion may be drawn that Chel 138 has no therapeutic value in experimentally induced manganese poisoning.

The authors wish to express their gratitude to Thomas Speckman for the use of the pressurized ionization chamber, and to William Westfall for assistance in calibration of the scintillation counter.

References

1. Fried, J., A. Lindenbaum, M. W. Rosenthal, and J. Schubert. Progress report: Radioelement removal. Quarterly Report of Biological and Medical Research Division, Argonne National Laboratory. ANL-5576, p. 98 (1956).
2. Lindenbaum, A., and J. Schubert. Progress report: Radioelement removal. II. This report, p. 130.
3. Kosai, M. F., and A. J. Boyle. Ethylenediaminetetraacetic acid in manganese poisoning of rats. *Ind. Med. and Surg.* 25: 1-3 (1956).
4. The Versenes. Sec. 4, p. 2. Bersworth Chemical Co., Framingham, Mass. (1954).
5. Maynard, L. S., and S. Fink. Influence of chelation on radiomanganese excretion in man and mouse. *J. Clin. Invest.* 35: 831-836 (1956).
6. ———, and G. C. Cotzias. The partition of manganese among organs and intracellular organelles of the rat. *J. Biol. Chem.* 214: 489 (1955).

137

PROGRESS REPORT: INTERACTIONS BETWEEN DIVALENT
TRANSITION METALS AND ORGANIC ANIONS. II.

Jack Schubert, William Westfall and Arthur Lindenbaum

A program previously described⁽¹⁾ for the determination of the formation quotient of complexes formed by divalent transition metals and organic anions is being continued. The $\log K_{\text{MnA}}$ values,* obtained by the ion exchange method** and uncorrected for ionization, hydrolysis or binding by other ligands, have now been determined for manganese (Mn^{++}) (Table 49).

TABLE 49

Log K_{MnA} values for manganese

Organic anion	$\log K_{\text{MnA}}$	Organic anion	$\log K_{\text{MnA}}$
Acetate	0.61	Fumarate	1.00
Glycollate	1.06	Aconitate (trans)	2.27
Lactate	1.19	Aconitate (cis)	2.47
Sulfosalicylate	1.35	Malate	2.24
3-Indolylacetate	0.52	Oxalacetate	1.68
Malonate	2.32	Citraconate	1.76
Succinate	1.27	Biaspartate	1.96
Glutarate	1.13	Tricarballylate	1.99
Pimelate	1.08	Citrate	3.55
Azelate	1.03	Isocitrate	2.55
Maleate	1.69	α -Carboxy pimelate	1.91

The binding of Mn^{++} and Zn^{++} with citrate has been determined, in addition, by a more exact titrimetric method.⁽²⁾ The values of $\log k_1$ obtained for the 1:1 complex were 3.53 for Mn^{++} and 4.75 for Zn^{++} , both in excellent agreement with values obtained by the ion exchange method.

References

- Schubert, J., W. Westfall, and A. Lindenbaum. Progress report: Interactions between divalent transition metals and organic anions. Quarterly Report of Biological and Medical Research Division, Argonne National Laboratory. ANL-5518, p. 208 (1956).
- Li, N. C., O. Gawron, and G. Bascuas. Stability of zinc complexes with glutathione and oxidized glutathione. J. Am. Chem. Soc., 76:225-229 (1954).

*In the reaction $\text{M} + \text{A} = \text{MA}$; $K_{\text{MA}} = \frac{(\text{MA})}{(\text{M})(\text{A})}$ where M is a divalent cation and A is a ligand.

**Ionic strength (μ) = 0.16 in Na^+ ; pH = 7.25 in veronal buffer; T = $25 \pm 1^\circ \text{C}$.

SOLUBILITY MEASUREMENTS OF CH₄, N₂, CO, CO₂, AND C₄F₈ IN
VARIOUS MATERIALS OF INTEREST TO BE USED IN IONIZATION
CHAMBERS FOR NEUTRON DOSIMETRY

S. S. Brar and L. D. Marinelli

Since, at present, practical neutron dosimetry depends on gas ionization, it is important that the tissue equivalent gas mixture used in tissue equivalent chambers be stable in time because of the absorption of the gas filling by the chamber walls. Consequently, the solubility of the above-mentioned gases in a few commonly employed chamber materials has been measured and the results are presented in tabular form.

TABLE 50

Solubility factors

Gas	Solubility factor, α^*			
	Polystyrene	Polyethylene	Markite**	Graphite
C ₄ F ₈		0.10	0.13	0.30
CO ₂	2.0	0.36	0.35	0.054
CO	0.14	0.16	0.15	0.006
CH ₄	0.32	0.17	0.17	0.025
N ₂	0.05	0.11	0.090	0.017

$$* \alpha = \frac{\text{Number of atoms of dissolved gas/cc of solid in equilibrium at } 25^{\circ}\text{C}}{\text{Number of gas atoms/cc of gas}}$$

**Markite Company, 155 Waverly Place, New York City, N.Y.

SERUM GLUTAMIC OXALACETIC TRANSAMINASE ACTIVITY AS AN INDEX OF LIVER CELL INJURY DUE TO BERYLLIUM POISONING

Asher J. Finkel and Dorice Czajka

Glutamic oxalacetic transaminase, which catalyzes the reversible transfer of an amino group from glutamic to oxalacetic acid, is widely distributed in animal tissues. The greatest concentration is in heart muscle with skeletal muscle, brain, liver, and kidney following in decreasing order. LaDue *et al.*⁽¹⁾ noted an immediate marked increase in serum glutamic oxalacetic transaminase (SGO-T) activity after myocardial infarction and found a correlation between the height and duration of SGO-T elevation and the degree of myocardial injury. More recently, Wroblewski and LaDue⁽²⁾ found that elevation of SGO-T activity in acute liver cell injury was an indication of cell destruction although not of liver function.

In view of the interest in experimental beryllium poisoning in animals, we thought it might be of value to follow the SGO-T changes in mice given intravenous injections of several dosages of beryllium sulfate since this compound is known to produce focal and mid-zonal necrosis of mouse livers.

Experimental Procedure

Groups of CF No. 1 female mice were injected intravenously with 0.5, 0.4, 0.3, 0.2, and 0.1 mg Be/kg given as BeSO₄. The LD₅₀ of Be for mice is approximately 0.5 mg Be/kg under these conditions. Three groups of controls were prepared: the first was given distilled water, the second Na₂SO₄ equimolar with 0.5 mg Be/kg; the third group received no injection. Each injection consisted of a volume equal to 0.01 ml/g body weight. Blood was removed by cardiac puncture after the mouse was anesthetized with ether, and the thorax opened. The serum was stored at -8.5°C; Wroblewski has shown that the SGO-T value does not change when serum is stored frozen for a period up to 3 weeks. SGO-T determinations were made according to Steinberg's modification⁽³⁾ of the method of Wroblewski and LaDue with the exception that smaller blood volumes were used, since the normal mouse SGO-T values are approximately two to five times higher than those of man.

The livers were removed for histological examination. The histopathology of these sections will be reported at a later date and correlated with the SGO-T values.

Results and Discussion

Table 51 and Figure 38 indicate that in general the level of SGO-T is correlated with the level of beryllium injected. This situation is readily apparent in the data for the first and fourth days after injection, and is only slightly obscured for the intervening days at the higher dose levels where the mean SGO-T values show some irregularity. The latter results can be attributed not only to the variability and unusual height of the SGO-T values but more importantly to the fact that some of the mice at these toxic dose levels died before the day of scheduled sacrifice so that the less sick animals were sampled. The three control groups gave results that are indistinguishable from each other, and the grand mean of these groups, 72.6 ± 28 (S.D.), is in agreement with published results for normal mice. The SGO-T values for 0.1 mg Be/kg also fall within a range of two standard deviations from the control mean, as do the values for the 0.2 mg Be/kg group with the exception of those for the third day. The SGO-T values show a marked decline toward normal levels by the fourth day. This result is consistent with the experience that most mice that die from experimental beryllium poisoning succumb by the third day after receiving a toxic intravenous dose of BeSO_4 .

TABLE 51

Serum glutamic oxalacetic transaminase (SGO-T) values
in mice given intravenous BeSO_4

	Day 1		Day 2		Day 3		Day 4	
	n*	Mean**	n	Mean	n	Mean	n	Mean
Beryllium-treated:								
0.5 mg/kg	6	660	8	1551	6	1847	2	378
0.4 mg/kg	4	562	4	4459	4	566	3	136
0.3 mg/kg	5	262	6	1542	5	813	5	111
0.2 mg/kg	4	115	4	120	4	188	4	103
0.1 mg/kg	3	99	3	68	3	80	3	70
Controls:								
Na_2SO_4	5	59	5	85	11	71	2	70
Untreated	3	67	3	57	3	81	3	122
Distilled water	2	74	2	80	3	57	3	93

Grand mean, controls: 72.6 ± 28 (S.D.)

*Number of animals

**SGO-T values are arbitrary units after the method of Wroblewski and LaDue.⁽²⁾

Figure 38

Serum glutamic oxalacetic transaminase activity in mice previously injected intravenously with beryllium sulfate. Beryllium dosages are given in mg Be/kg. The normal range of SGO-T values in mice extends from 17 to 129 units, with a mean value of 73 units.

Summary

Mouse serum glutamic oxalacetic transaminase (SGO-T) was used to establish the lowest level of intravenously injected beryllium sulfate that would produce a hepatotoxic effect. This level was found to lie between 0.1 and 0.2 mg Be/kg given as BeSO_4 .

References

1. LaDue, J. S., Wroblewski, F. and Karmen, A. Serum glutamic oxalacetic transaminase activity in human acute transmural myocardial infarction. *Science* 120: 497-499 (1954).
2. Wroblewski, F. and J. LaDue. Serum glutamic oxalacetic transaminase activity as an index of liver cell injury: A preliminary report. *Annals of Int. Med.* 43: 345-360 (1955).
3. Steinberg, D., D. Baldwin and B. Ostrow. A clinical method for the assay of serum glutamic-oxalacetic transaminase. *J. Lab. Clin. Med.* 48: 144-151 (1956).