ARGONNE NATIONAL LABORATORY
9700 South Cass Avenue
Argonne, Illinois 60440

BIOLOGICAL AND MEDICAL RESEARCH DIVISION
SEMIANNUAL REPORT

July through December 1962

October 1963

Preceding Report:
ANL-6723 January through June 1962

Operated by The University of Chicago
under
Contract W-31-109-eng-38
with the
U. S. Atomic Energy Commission

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>
Fractionated, low dose-rate irradiation of mice with fission neutrons and Co^{60} gamma-rays Howard H. Vogel, Jr. and Donn L. Jordan	5
Cell division in the small intestine of rats exposed to continuous gamma irradiation Samuel Leshner, Leonard F. Lamerton, R. J. Michael Fry and George A. Sacher	8
Self-absorption and reciprocity law in radiation dosimetry: calculation of absorption coefficient from measured intensities of radiation Ernesto Trucco	11
Attempts to establish immune tolerance by massive organ transplantation Bernard N. Jaroslow, Thomas G. Baffes and F. H. Ketola . .	15
Progress report: Monte Carlo calculations in the stochastic theory of mortality George A. Sacher and Ernesto Trucco	20
An appraisal of differences in acute mortality from X- or gamma radiation between protracted, split, and fractionated exposures Sylvanus A. Tyler and S. Phyllis Stearner	21
The toxicity of Ra^{226} in mice: A progress report Miriam P. Finkel, Patricia B. Jenkins and Birute O. Biskis	26
Toxicity and metabolism of radionuclides. IV. Progress report for period ending December 31, 1962 William P. Norris, Louise S. Lombard, Carl E. Rehfeld and Thomas W. Speckman	31
Progress report: Tumor development following injection of carcinogens at multiple sites Austin M. Brues, Harry Auerbach and Georgia DeRoche . .	49
Dynamics of tumor growth Anna Kane Laird	57
Physiological effects of glucose oxidase in mice Robert N. Feinstein, Marilyn Coulon and John E. Seaholm . .	63

TABLE OF CONTENTS

	<u>Page</u>
Effects of histological and histochemical procedures on the intensity of the label in autoradiograms of cells labeled with tritiated compounds Renato Baserga and Walter E. Kisielewski	73
Some comments concerning the feasibility of determining the radiation-induced mutation rate for sex-linked recessive lethals in the mouse Douglas Grahn and Katherine F. Hamilton.	84
Progress report: Plutonium removal, tumor incidence studies. IV. Marcia W. Rosenthal, Joan F. Markley and Arthur Lindenbaum.	87
Progress report: Plutonium removal. VI. Chemical removal of marrow and its associated plutonium from bone Marcia W. Rosenthal, Joan F. Markley and Arthur Lindenbaum.	92
Mucopolysaccharides and mucoproteins of ossifying cartilage Arthur Lindenbaum.	94
Mucoprotein extracts of ossifying cartilage Klaus E. Kuettner and Arthur Lindenbaum	95
Golgi fine structure in a radiosensitive giant amoeba Edward W. Daniels and Nancy Reed.	96
Extraction of DNA and RNA from radiosensitive amoebae and the use of these compounds in radio restoration and transplantation experiments Edward W. Daniels, Evelyn P. Brown and Anna K. Laird. . .	97
Fractionation of amoebae in various media Edward W. Daniels, Evelyn P. Brown and Y. E. Rahman . . .	101
Progress report: The biosynthesis of methionine. Utilization of S-adenosylmethionine for the biosynthesis of methionine Stanley K. Shapiro, Phoebe Lohmar and Mary Hertenstein . .	113
Progress report: Biosynthesis of methionine from homocysteine and sulfonium derivatives in <u>Escherichia coli</u> . II. Morris Pfeffer.	122

TABLE OF CONTENTS

	<u>Page</u>
Ultraviolet microscopy of purine compounds in the yeast vacuole George Svihla, Julia L. Dainko and F. Schlenk	126
Ionic equilibria of the nucleosides Lyle G. Bunville.	137
Progress report: Soluble proteins from cell nuclei A. D. Barton	138
Phosphate esterification and hydrolysis in mitochondrial preparations of normal and X-irradiated rat spleen John F. Thomson	145
Fragility studies of rat spleen lysosomes after X-irradiation Y. E. Rahman	150
Spontaneous non-lethal damage in a "small" clone of Chinese hamster cells in culture Warren K. Sinclair and Carolan H. Wieker	156
Chromosome number and aberrations in "large" and "small" clones of irradiated Chinese hamster cells in culture Warren K. Sinclair and Agnes N. Stroud	161
Spontaneous chromosome aberrations and the nature of near- diploid cells of Chinese hamster (<u>Cricetulus griseus</u>) in vitro C. K. Yu	171
Preliminary experiments with the "sponge technique" for electron microscopic studies of fibrillogenesis in the regenerating tendon F. Wassermann	184
Preliminary experiments on labeling of grasshopper sperma- togonia with tritiated thymidine Theodore N. Tahmisian and Rosemarie L. Devine	192
An examination of embryological and myogenetic development in the zebra fish, <u>Brachydanio rerio</u> (Hamilton-Buchanan) Theodore N. Tahmisian, Betty Jean Wright and Bronson R. Hall	194

TABLE OF CONTENTS

	<u>Page</u>
Metabolic bases of the impairment of root initiation by ionizing radiation	
Solon A. Gordon, Lowell Woodstock and Bhagwan K. Gaur . .	205
X-radiation response of auxin formation by animal tissue	
Solon A. Gordon and Evelyn Buess.	211
Spectrophotometric method for the determination of lipoxidase activity	
Kenneth Surrey	221
Progress report: The effect of X-rays on the formation of flower primordia in Xanthium	
William Chorney	228
Progress report: The boron requirement of <u>Chlorella vulgaris</u>	
Landy J. McBride.	230
Publications	233

FRACTIONATED, LOW DOSE-RATE IRRADIATION OF MICE WITH FISSION NEUTRONS AND Co^{60} GAMMA-RAYS

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

The radiation exposures reported here were designed to provide additional data on the comparative lethal effects of fission neutrons and Co^{60} γ -rays in CF No. 1 female mice.

Under the conditions of exposure at CP-5, fission neutrons have been found to be almost three times as effective as Co^{60} γ -rays for acute 30-day lethality in this mouse. There is evidence, confirmed in several laboratories, that the relative biological effectiveness (RBE) of neutrons over γ -rays is increased from about 2 or 3 for single acute exposures to as high as 5 to 15 for irradiation with small doses over long periods of time.⁽¹⁾ In the present experiment the value of 5 was arbitrarily chosen for the dose ratio. Thus, the total weekly dose was 300 rads for γ -rays and 60 rads for neutrons. In each case the mice received 13 weekly doses, delivered in 1, 3, or 6 equal fractions per week. All animals were exposed in the gamma-neutron radiation chamber at the CP-5 reactor.⁽²⁾ Both radiations were delivered at a dose rate of approximately 1 rad/min. This experimental plan is summarized in Table 1.

TABLE 1

Survival at 280 days after first irradiation*

Radiation	No. of mice exposed	Exposures per week	Dose per exposure, rads	Total dose, rads	Mean survival time, days
Co^{60} γ -rays	24	1	300	3900	80.0
	24	3	100	3900	80.1
	24	6	50	3900	105.6
Fission neutrons	32	1	60	780	183.8 (29)**
	32	3	20	780	158.1 (29)
	32	6	10	780	158.8 (31)
Unirradiated controls	39	0	0	0	

*During this period 5% (2/39) unirradiated control mice died.

**Figures in parentheses give the numbers of deaths up to 280 days after first irradiation.

The data are not yet complete since a few neutron-irradiated mice are still alive. However, several general conclusions are already clear. Survival data (Table 1) indicate that the mean survival times of the gamma-irradiated mice are not significantly different whether they are exposed once or three times a week. There is a suggestion that daily exposure (six per week) increases the mean survival time, but the numbers are small and the difference may not be significant. There is no such indication for the neutron-irradiated mice.

Survival times for the three sub-groups for each radiation appeared similar; hence, the data were pooled and cumulative mortalities for the two radiations were compared (Figure 1). It is clear that under the conditions of this experiment the neutron/gamma RBE is less than 5. Although these exposures were carried out over a 13-week period, the RBE appears

to resemble that for acute exposures rather than the elevated value associated with duration-of-life exposure to small daily doses. Since the intensity (1 rad/min) may be too high, we have replicated this experiment at still lower dose rates; such data both for fractionation and protraction of dose are expected to be useful in planning neutron exposures at JANUS.

Figure 1. Comparison of cumulative mortality in neutron-irradiated and γ -irradiated mice

they do not survive through the long latent period to develop lymphomas. Our replicated experiment has been set up with a dose ratio of 3 rather than 5; this should yield comparable effects on survival, and we should be able to determine whether there is a real difference in lymphoma incidence following these two radiations.

The thymic lymphoma incidence observed at autopsy in the irradiated mice is given in Table 2. This reticular tumor of the thymus gland is the leading cause of death in the neutron-irradiated mice, but its incidence is much lower in the mice exposed to γ -rays. It is probable that this difference is largely an effect of the different times of mortality: since the γ -irradiated mice die much earlier,

TABLE 2

Incidence of thymic lymphomas (observed at autopsy)

Radiation	Exposures per week	No. of mice with lymphomas	Incidence, %
		No. of mice observed	
Co ⁶⁰ γ -rays	1	5/24	21
	3	2/24	8
	6	7/23	30
	Total	14/71	20
Fission neutrons	1	20/28	71
	3	19/29	66
	6	25/31	81
	Total	64/88	72

References

1. Neary, G. J., R. J. Munson, and R. H. Mole. Chronic Radiation Hazards. An Experimental Study with Fast Neutrons. Pergamon Press, New York, 1957.
2. Vogel, H. H., Jr., R. A. Blomgren, and N. J. G. Bohlin. Gamma-neutron radiation chamber for radiobiological studies. Nucleonics 11 (3), 28-31 (1953).

CELL DIVISION IN THE SMALL INTESTINE OF RATS EXPOSED TO CONTINUOUS GAMMA IRRADIATION

Samuel Leshner, Leonard F. Lamerton, R J Michael Fry
and George A. Sacher

Experimental evidence from several sources suggests that all cells, regardless of their state at the time of exposure, are to some degree affected by irradiation, but the degree of sensitivity is dependent upon the stage of the generation cycle at the time of irradiation.⁽¹⁻³⁾ In a rapidly dividing cell population the generation cycle is short and a greater number of cells are in the more sensitive phases of the cycle, therefore, highly proliferative organs are more heavily damaged by irradiation. Considerable amounts of data are accumulating concerning the effects of acute single exposures on all stages of the generation cycle of rapidly dividing cells, but little is known concerning the effects of low-dose, long-protracted exposures. It is thought that cells react differently to prolonged exposures to ionizing radiations than they do to single or fractionated exposures.⁽⁴⁻⁶⁾

In an earlier series of experiments⁽⁵⁾ it was found that in mice exposed to 12 r per day for 200 days the proliferative cells of the duodenum had apparently adapted very effectively to this daily stress and were producing new cells in an efficient manner and at an even more rapid rate. In this report preliminary data are presented concerning the effects of continuous γ -irradiation on the generation cycle of the crypts of the rat small intestine.

Male and female AMF₁ rats (August females x Marshall males) were placed in a Cs¹³⁷ exposure unit of approximately 6 curies at six weeks of age. They were caged individually and fed a balanced diet known as M.R.C. diet No. 41 B. Temperature was maintained at about 70°F. Animals were exposed over a 23.5-hr day. The source was lowered into a shield at about the same time each day so that food and water supplies could be replenished.

Three experimental groups were put into the irradiation chamber to receive 50 rads per day for 12, 35, or 105 days. At the end of the exposure period each rat was given 100 μ c of tritiated thymidine (Schwartz Laboratories, 360 mc per M) by intraperitoneal injection and returned to the caesium unit. Animals were then sacrificed at closely-spaced intervals from $\frac{1}{2}$ hour to 30 hours after injection. Two groups of control animals, 54 and 147 days of age, were similarly treated.

Three segments of the upper small intestine 3 cm below the pylorus were removed, fixed, dehydrated and embedded in paraffin; 3- μ sections

were prestained by the Feulgen reaction and autoradiographs were prepared. Generation time was estimated from the percentage of metaphase and anaphase mitotic figures labeled at each time interval. Other determinations to be made include: 1) number and position of labeled nuclei at 1 hr, to establish a labeling index (LI); 2) number of labeled nuclei at hourly intervals up to 8 hr, to obtain an estimate of cell production; 3) number of mitotic figures at hourly intervals up to 8 hr (MI); 4) concentration of Feulgen-positive karyolytic bodies per crypt for each rat; 5) ratio of aberrant to normal anaphase mitotic figures; and 6) total crypt cell population in irradiated and unirradiated animals.

When the percentage of mitotic figures labeled was plotted against time (Figures 2 and 3) cyclic curves were obtained for all five groups. The following discussion is tentative, and the values plotted in these curves will be supported by additional determinations made on randomly sorted slides. Some interpretations can be made, since in previous experiments it has been shown that the shape and general position of the curves based on per cent labeled mitotic figures can be established by scoring a minimum of 100 metaphase and anaphase figures per animal. However, definitive interpretations should not be based on determinations of this magnitude.

Examination of the curves shows that differences between irradiated and unirradiated rats are quite small. The minimum generation time, which is the time interval between the start of the two successive ascending arms, is the same in rats irradiated for 12 and 35 days and in the 54-day-old unirradiated animals; but it appears to be an hour longer in the group exposed for 105 days and even longer in the 147-day-old unirradiated controls. Postsynthesis (G_2) plus mitosis (M) is extremely rapid in the two control groups and the 105-day irradiated animals ($1\frac{1}{2}$ hours) but

Figure 2. The percentage of mitotic figures (metaphase plus anaphase) labeled versus time after injection of tritiated thymidine, in unirradiated rats 54 days old (left) and 147 days old (right) at the time of injection.

Figure 3. The percentage of mitotic figures (metaphase plus anaphase) labeled versus time after injection of tritiated thymidine in rats irradiated with Cs^{137} γ -rays. The rats were first exposed to 50 rads per day for 12, 35, or 105 days. The label was injected after this exposure period and the animals were returned to the exposure chamber.

increases to 2 hours in the 12 and 35 day exposure groups. The synthesis period (S) seems to be essentially the same in all 5 groups although the minimal synthesis interval is quite different in the irradiated animals. Finally in the control animals there is a clear deterioration of the second cycle which is not seen in the irradiated animals; this must be interpreted as a toxicity factor.

References

1. Dewey, W. C., and R. M. Humphrey. Relative radiosensitivity of different phases in the life cycle of L-P59 mouse fibroblasts and ascites tumor cells. *Radiation Res.* 16, 503-530 (1960).
2. Whitfield, J. F., and R. H. Rixon. Effects of x-radiation on multiplication and nucleic acid synthesis in cultures of L-strain mouse cells. *Exptl. Cell Res.* 18, 126-137 (1959).
3. Whitmore, G. F., C. P. Stanners, J. E. Till, and S. Gulyas. Nucleic acid synthesis and the division cycle in x-irradiated L-strain mouse cells. *Biochim. Biophys. Acta.* 47, 66-77 (1961).
4. Quastler, H., J. P. M. Bensted, L. F. Lamerton, and S. M. Simpson. II. Adaptation to continuous irradiation: Observations on the rat intestine. *Brit J. Radiol.* 32, 501-512 (1959).
5. Leshner, S., R. J. M. Fry, and G. A. Sacher. Effects of chronic gamma irradiation on the generation cycle of the mouse duodenum. *Exptl. Cell Res.* 25, 398-404 (1961).
6. Fry, R. J. M., S. Leshner, A. Sallese, and E. Staffeldt. The generation cycle of the mouse duodenal crypt cells exposed to 220 r per day Co^{60} gamma irradiation. *Radiation Res.* 19, 628-635 (1963).

SELF-ABSORPTION AND RECIPROCITY LAW IN RADIATION
DOSIMETRY: CALCULATION OF ABSORPTION
COEFFICIENT FROM MEASURED
INTENSITIES OF RADIATION

Ernesto Trucco

I. In a previous report⁽¹⁾ we had derived the relation

$$k = F(z, \rho) \equiv \frac{2 \int_{t=0}^1 \left[1 - \sqrt{1 + z^2(t^2 - 1)} \right] e^{-\rho t} dt}{\left[1 - \frac{(1 - z^2)}{2z} \ln \left(\frac{1+z}{1-z} \right) \right]}, \quad (1)$$

which is Equation (19) of reference 1. We assume the reader is familiar with that report and will refer to it for the meaning of symbols appearing in the above equation. A computer program was set up by Mr. Anthony J. Strecok of the Applied Mathematics Division a) to calculate $F(z, \rho)$ as a function of z and ρ , and b) to solve Equation (1) for ρ when k and z are given.

It should be noticed that $F(z, \rho) \geq 0$, $F(z, 0) = 1$, and

$$(-1)^n \frac{\partial^n F(z, \rho)}{\partial \rho^n} = \frac{2 \int_{t=0}^1 t^n \left[1 - \sqrt{1 + z^2(t^2 - 1)} \right] e^{-\rho t} dt}{\left[1 - \frac{(1 - z^2)}{2z} \ln \left(\frac{1+z}{1-z} \right) \right]} > 0.$$

Thus, for fixed $z > 0$, $F(z, \rho)$ is a monotonically decreasing function of ρ , so that the equation $k = F(z, \rho)$ always has exactly one solution, $\rho = \rho_0$, when $0 < k < 1$ and $z > 0$. If z is very small the calculation of ρ_0 requires careful programming since both the numerator and the denominator in the right-hand side of Equation (1) tend to zero for $z \rightarrow 0$. But, as we have seen,⁽¹⁾ the relation

$$\lim_{z \rightarrow 0} F(z, \rho) = v(\rho) \equiv \frac{3}{\rho^3} \left[-1 + \frac{\rho^2}{2} + (\rho + 1) e^{-\rho} \right]$$

is useful in this case.

Lack of space prevents reproducing all of Strecok's calculations. However, in Table 3 the function $F(z, \rho)$ is given for a few arbitrarily selected values of z and ρ . [The program printed results with eight decimal figures (the last of which may not always be correct) although such accuracy is not usually required for practical application.]

TABLE 3

 $F(z, \rho)$ for a few values of z and ρ

z	ρ			
	0.01	0.05	0.10	0.30
0.01	0.99626002	0.98149750	0.96347968	0.89596556
0.05	0.99626029	0.98149895	0.96348250	0.89597341
0.10	0.99626122	0.98150355	0.96349152	0.89599814
0.30	0.99627172	0.98155502	0.96359213	0.89627409
0.50	0.99629563	0.98167268	0.96382227	0.89690582
0.70	0.99634265	0.98190355	0.96427393	0.89814774
0.90	0.99645255	0.98244378	0.96533187	0.90106760

z	ρ			
	0.50	1.00	1.50	2.00
0.01	0.83510427	0.70727748	0.60695692	0.52725327
0.05	0.83511625	0.70729687	0.60698065	0.52727937
0.10	0.83515397	0.70735782	0.60705526	0.52736136
0.30	0.83557521	0.70803886	0.60788937	0.52827822
0.50	0.83654015	0.70960186	0.60980694	0.53038941
0.70	0.83844038	0.71269160	0.61361129	0.53459264
0.90	0.84292436	0.72004901	0.62274997	0.54477333

II. The ratio k was determined experimentally by Sinclair^(2,3) who studied its dependence on D (diameter of sphere) and g (distance between point P and center of sphere) for a material with unknown absorption coefficient μ . From k and z , which is equal to $D/2g$, we can calculate ρ_0 by means of Equation (1). This, then, gives us the value of μ , since $\mu = \rho_0/D$.

In all of Sinclair's experiments z was less than 0.1, its largest value being, in fact, equal to 0.094 for $D = 18.8$ cm and $g = 100$ cm. Under these circumstances we may expect that $k \approx v(\rho)$ to a very good approximation, and this was indeed the case. The error made in replacing z by zero turned out to be completely negligible as compared to experimental uncertainties in the measurement of k .

Table 4 gives some of the k values found by Sinclair (corrected to make $k = 1$ when $\rho = 0$), and the corresponding values of μ calculated from Equation (1).

III. In Table 5 the data were replaced by fictitious numbers such that, for fixed k and D , the actual value of g (or z), as given in Table 4, was either divided or multiplied by three.

TABLE 4

Values of k found by Sinclair, corrected to make $k = 1$ when $\rho = 0$, and corresponding values of μ calculated from Equation 1.

D, cm	g, cm	k	$100 \times \mu_{\text{calc}},$ cm^{-1}	D, cm	g, cm	k	$100 \times \mu_{\text{calc}},$ cm^{-1}
4.0	100	0.940	4.1801255	4.0	300	0.951	3.3855636
7.0	"	0.890	4.5532862	7.0	"	0.907	3.7978568
12.0	"	0.849	3.7712534	12.0	"	0.848	3.7990079
16.0	"	0.815	3.5684427	16.0	"	0.819	3.4783853
18.8	"	0.784	3.6460728	18.8	"	0.790	3.5244977

TABLE 5

Values of D and k are as in Table 4, but g values have been either multiplied or divided by three

D, cm	g, cm	k	$100 \times \mu_{\text{calc}},$ cm^{-1}	D, cm	g, cm	k	$100 \times \mu_{\text{calc}},$ cm^{-1}
4.0	300	0.940	4.1800752	4.0	100	0.951	3.3856057
7.0	"	0.890	4.5531170	7.0	"	0.907	3.7979977
12.0	"	0.849	3.7708372	12.0	"	0.848	3.7994275
16.0	"	0.815	3.5677347	16.0	"	0.819	3.4790747
18.8	"	0.784	3.6450638	18.8	"	0.790	3.5254712

We see that, all other things being equal, μ_{calc} has almost exactly the same value for $g = 100$ cm and for $g = 300$ cm, both of these being practically equivalent to $g = \infty$ if D is less than 20 cm.

On the other hand, when D and g are given, a relatively small variation of k , such as might be produced by experimental errors of measurement, will cause a marked change in μ_{calc} .

It is quite reasonable, therefore, to follow Sinclair's procedure, i.e., use the simple equation $k = v(\rho)$, taking k to be the average of the two values found for $g = 100$ cm and $g = 300$ cm.

Dr. Sinclair's permission to use his data, and the assistance of Mr. Strecok in performing the calculations, are gratefully acknowledged.

References

1. Trucco, E. Progress Report: A note on self-absorption and reciprocity law in radiation dosimetry. Biological and Medical Research Division Semiannual Report, January through June 1962. Argonne National Laboratory, ANL-6723, pp. 16-27.
2. Sinclair, W. K. Absorbed dose in externally irradiated biological specimens. I. Co⁶⁰ gamma-radiation. Biological and Medical Research Division Semiannual Report, January through June 1961. Argonne National Laboratory, ANL-6464, pp. 133-142.
3. Sinclair, W. K. Absorbed dose in biological specimens irradiated externally with cobalt-60 gamma-radiation. Radiation Res. 20, 288-297 (October 1963).

ATTEMPTS TO ESTABLISH IMMUNE TOLERANCE BY MASSIVE ORGAN TRANSPLANTATION

Bernard N. Jaroslow, Thomas G. Baffes
and F. H. Ketola

In this study we hoped to test the feasibility of a hypothesis which suggests that organ transplantation can be successfully achieved if the transplant is so large as to expose the recipient to continuous massive doses of antigen. An analogous situation was created by Felton.⁽¹⁾ He found that mice produced antibody to small doses of pneumococcal polysaccharide, but large doses of the antigen resulted in increased susceptibility to pneumococcal infection, i.e., pneumococci were not destroyed by circulating antibody. If enough antigenic material, i.e., organs and tissues, can be transferred to establish and maintain immune tolerance in animals that are physiologically compatible, then the transplanted organs might survive. We know that parabiosis, in which the above situation is created, can be successfully established.

The work reported here is based on the following hypothesis outlined in Figure 4. In the adult animal there is a stem cell A, which does not respond to antigenic stimulation, but does divide and mature into type B. The B cell upon contact with an antigen is altered in such a way that it and its descendents are rendered incapable of responding to that antigen. If it is not desensitized by an antigen, it proliferates and matures into C cells. The C cell, when stimulated or sensitized by antigen, proliferates, and differentiates into D cells, which produce antibody for a limited time. A system similar to this was proposed by Medawar.⁽²⁾

Figure 4. The proposed scheme for maturation of the cells of the antibody-forming system and their response to antigen.

There is supporting evidence for such a scheme. The D cells were definitively shown by Nossal⁽³⁾ to be plasma cells; he isolated single cells from an immunized donor in droplets of saline, and found that only cells of the plasma cell series produced antibody to bacteria. Large lymphocytes are probably the C cells; as described by Taliaferro and Taliaferro,⁽⁴⁾ they can phagocytize particulate antigen, they have a dense basophilic cytoplasm

and they can mature into plasma cells. The B cells are those present in immunologically immature animals; the phenomenon of immune tolerance, first experimentally demonstrated by Billingham et al.,⁽⁵⁾ indicates that contact of these cells with antigen renders their descendants in the C category specifically unresponsive to an antigen. The demonstration by Smith and Bridges⁽⁶⁾ that immune tolerance is only maintained in the presence of antigen indicates that a new population of B cells matures into C cells when antigen disappears. It would seem, therefore, that there must be a continuous generative source of cells in the adult, namely, the stem (A) cells.

In parabiosis between immunologically mature animals, the massive production of circulating antigen might saturate cell types B and C; the type C cells already present would then mature and the resulting D cells would produce antibody. If sufficient circulating antigen were present, the amount of antibody reaching the target organ might be insufficient to induce extensive injury. After a time antibody production would decline and then cease; the production of new C cells would cease if the population of B cells were kept saturated with antigen. In this manner, the state of immune tolerance could be established and maintained because no new C cells would develop. If on the other hand, the presence of large amounts of circulating antigen inhibits the synthesis and release of antibodies by the C cells, i e. true immune paralysis, then the establishment and maintenance of the transplant could be accomplished with less injury. This latter possibility is considered since Sercarz and Coons⁽⁷⁾ demonstrated that no free antibody was produced in mice that were injected with large amounts of BSA. Only after BSA finally disappeared from the circulation did the mice produce antibodies to BSA, and they did this without further antigenic stimulation.

Organ transplantation between members of a parabiotic pair, followed by separation should result in delayed, but eventual rejection of the transplanted organ. This was demonstrated in Kamrin's⁽⁸⁾ study in which he made reciprocal homografts of wedges of kidney into the kidney of the parabiotic partner. After separation of the two rats, the transplants were found to have survived 14 to 21 days as opposed to 7 to 11 days for non-parabiotic controls. Presumably, as circulating antigen disappeared, B cells in increasing numbers escaped contact with antigen and matured into C cells which then matured and synthesized antibody. In time, enough antibody reached the graft and it was rejected.

Where homotransplantation has been successful without the use of radiation or drug therapy, it is based on the techniques and principles of immune tolerance as first described by Billingham et al.⁽⁵⁾ In the prenatal or neonatal animal, C cells are not present and have no chance to develop. As a result less antigen is needed to maintain immune paralysis.

In essence, we are trying to create a situation in which the transplant can induce immune tolerance and maintain it by producing enough

circulating antigen to prevent the initial surge of antibodies from causing irreversible injury and to stop the production of new antibody-forming cells.

In our test of the hypothesis, we hoped to establish immune tolerance through parabiosis and to maintain it, after separation, by transplants of several organs.

Methods and Results

We used beagles or pointers chosen so that the partners were of the same sex, and, within five pounds, of the same weight.

To establish parabiosis both aortas were sectioned below the renal arteries and the anterior end of one was connected to the posterior end of the other with woven teflon grafts. We used a dorsolateral approach and dissected the aortas free without entering the peritoneum. The grafts passed through a pedicle of skin and the animals were partially immobilized in a joint body cast. This procedure is similar to that described by Rowe et al (9)

Ten operations were performed but only 3 pairs survived over 7 days, approximately the time of onset of immune rejection. One pair of beagles that survived 15 days was killed when the wound became grossly infected; all the limbs were edematous. A pair of pointers that survived 10 days developed edema in the limbs and when one died, the other exsanguinated into it. There was much less infection around the wound, but it had not begun to heal. In another pair of pointers that survived 15 days the course was the same except that healing of the incision was well underway.

Although spleens and lymph nodes showed extensive cellular proliferation, none of the pathologic changes appeared to be due to an immune response. Healing around the anastomoses was good and a pseudomembrane developed in the teflon graft. In all three cases, the graft was patent at death.

Because of the difficulties involved in trying to maintain dogs in parabiosis for extended periods, we are presently trying another procedure which may accomplish the same thing. We are transferring abdominal viscera by means of a modified Rokitansky procedure. In this maneuver the aorta, the vena cava, and the viscera are separated from all nerve and blood connections to the musculature; the portal vein, the vena cava, the aorta, the duodenum, the colon and the bladder are sectioned, and the abdominal viscera are moved into the abdominal cavity of a recipient from which all the abdominal organs except for liver and stomach had been removed.

To determine the physiological feasibility of the procedure we performed part of this operation on a single dog. All blood and nerve connections with the musculature were sectioned, the phrenic and vagus nerves were cut where they came through the diaphragm, and a pyloroplasty was performed. This dog, a beagle, is presently alive fourteen months after the operation, and in vigorous good health. The stools are entirely normal in appearance. The same operation was performed on a pointer except that the esophagus, the colon, the aorta and the vena cava were clamped for 35 minutes. No pyloroplasty was performed. This animal survived for six weeks; cause of death was volvulus of the intestine and herniation of the diaphragm.

On the basis of these two successes we felt that the operation was compatible with physiological requirements. Initially, we had also transferred liver, but in eight transfers the recipient died within 2 hr after circulation was restored. Death was due to anaphylactoid shock with the characteristic rapid decrease in blood pressure. The EKG showed changes attributable to anoxia but temporarily improved when benadryl and adrenaline were given.

In three other operations all of the viscera except the liver were transferred into a recipient that retained its own liver. These animals lived approximately 3, 7, and 9 hours respectively; none gave any indication of going into anaphylactoid shock during the first 2 hr as had all the others. On autopsy there was no observable pathologic change in the first two cases. In the third, death, which occurred 9 hours after surgery, was clearly due to frank hemorrhage. Thus, leaving the liver in the recipient animal was of definite value in avoiding anaphylactoid shock.

In two operations, we left the adrenal glands as well as the liver in the recipient. These animals lived 3 and $3\frac{1}{2}$ days respectively. The cause of death was uremia.

Acknowledgements

We wish to thank Miss Joan A. Stachura and Mr. Leo Norden for their technical assistance. We would also like to thank Mr. Durward Banister and Mr. William Hart for their special efforts during postoperative care of the dogs. We also wish to thank Swedish Covenant Hospital of Chicago for the use of some of their equipment.

References

1. Felton, L. The significance of antigen in animal tissues. *J. Immunol.* 61, 107-117 (1949).
2. Medawar, P. B. Theories of immunological tolerance. *Cellular Aspects of Immunity*, ed. G. E. W. Wolstenholme and M. O'Connor. Little, Brown and Co., Boston, 1959, pp. 134-149.
3. Nossal, G. J. V. Antibody production by single cells. III. The histology of antibody production. *Brit. J. Exptl. Pathol.* 40, 301-311 (1959).
4. Taliaferro, W. H., and L. G. Taliaferro. Reactions of the connective tissue in chickens to Plasmodium gallinaceum and Plasmodium lophurae. I. Histopathology during initial infections and superinfections. *J. Infect. Diseases* 97, 99-142 (1955).
5. Billingham, R. L., L. Brent, and P. B. Medawar. "Actively acquired tolerance" of foreign cells. *Nature* 172, 603-606 (1953).
6. Smith, R. T., and R. A. Bridges. Immunological unresponsiveness in rabbits produced by neonatal injection of defined antigens. *J. Exptl. Med.* 108, 227-250 (1958).
7. Sercarz, E. E., and A. H. Coons. The absence of antibody-producing cells during unresponsiveness to BSA in the mouse. *J. Immunol.* 90, 478-491 (1963).
8. Kamrin, B. B. Discussion of paper by Egdahl and Hume. *Ann. N. Y. Acad. Sci.* 64, 954-957 (1957).
9. Rowe, M. I., D. E. Martin, III, L. Tose, D. C. Nabseth, and R. A. Deterling, Jr. A new method of parabiosis in dogs utilizing a common channel with aortic cross circulation. *Surgical Forum* 12, 145-147 (1961).

PROGRESS REPORT: MONTE CARLO CALCULATIONS IN THE STOCHASTIC THEORY OF MORTALITY

George A. Sacher and Ernesto Trucco

A stochastic model has been presented to account for mortality in a large population composed of identical individuals. According to this theory⁽¹⁾ death occurs whenever random fluctuations of certain physiological variables carry a representative point outside of a "lethal boundary" in configuration space. These assumptions lead to a partial differential equation, called the Fokker-Planck equation, which must be solved with appropriate boundary conditions. As pointed out in a previous report,⁽²⁾ all purely analytical means for studying the solution of that basic equation have now been exhausted. A Monte Carlo program to investigate the escape of particles from a potential well was therefore set up, but this approach met with great difficulties when the probability of escape was very small, which is the case of greatest interest. Even the procedure of "forcing escapes" (page 59 of Reference 2) was not, in general, adequate. In order to obtain good results (i.e., reliable statistics) it seems necessary to choose the "forcing function" with great care. This problem is being studied by Dr. D. A. Woodward, of the Applied Mathematics Division, who has been able to achieve considerable progress in recent months.

In addition to the force given by the potential function, the particles are subject to a random force, $A(t)$, which may cause their escape from the well. $A(t)$ is not completely specified as a function of time; it may, on the contrary, be any one of a large family of functions. Woodward has chosen a special type, say $A_0(t)$, of force (i.e., one that might occasionally be realized and act on the particles). He has studied under what conditions a particle will escape when $A(t) = A_0(t)$. From this he is able to draw some conclusions which will guide him in selecting the "forcing function." Roughly speaking, an appropriate forcing function (not necessarily the only one) will be of the same type as $A_0(t)$ and such that, taken without additional random disturbances, it will almost (but not quite) cause a particle to escape from the well. Dr. Woodward's investigations are not yet completed and their details will be presented in a later report. Meanwhile we wish to express our thanks for his assistance in handling this difficult problem.

References

1. Sacher, G. A. and E. Trucco. The stochastic theory of mortality. Ann. N. Y. Acad. Sci. 96, 985-1007 (1962).
2. Sacher, G. A. and E. Trucco. Preliminary results from the Monte Carlo study in the stochastic theory of mortality. Biological and Medical Research Division Summary Report, January through December 1960, Argonne National Laboratory, ANL-6368, pp. 56-60.

AN APPRAISAL OF DIFFERENCES IN ACUTE MORTALITY FROM X- OR GAMMA RADIATION BETWEEN PROTRACTED, SPLIT, AND FRACTIONATED EXPOSURES

Sylvanus A. Tyler and S. Phyllis Stearner

To explain the acute mortality produced by a single, protracted exposure to X- or gamma radiation, a kinetic model of injury accumulation was proposed.⁽¹⁾ In essence, this model states that

Radiation initiates in an organism several deleterious effects that accumulate independently and that individually can cause death within 30 days after exposure. Death occurs when any single effect exceeds the critical level (or lethal bound) associated with that effect. Both time-dependent (reversible) and time-independent (irreversible) injury processes govern the accumulation of these effects. Time-dependent effects of prominence in acute lethality are reversed at a constant rate (linear reversal) during an exposure; i.e., dose effectiveness is reduced at a constant rate.

The model has been used to advantage in the statistical description of acute mortality patterns in the young chick,^(1,2) chick embryo,⁽³⁾ parakeet,⁽⁴⁾ pigeon,⁽⁴⁾ and mouse.⁽⁵⁾ Other investigators have accepted the postulation of an injury mechanism that is governed by a linear reversal process.⁽⁶⁾

The question now is whether this model can also serve as a conceptual framework for the observed acute mortality from split or fractionated exposures. Brown, Corp, and Mole⁽⁶⁾ recently demonstrated marked differences in acute radiation lethality between protracted and split exposures. These investigators found that the $LD_{50/30}$ increases at approximately the same constant rate when the exposure is given continuously for periods up to 6 hr and when it is fractionated or split within 6 hr of the conditioning dose. For intervals intermediate between 6 and 36 hr, split doses consistently gave lower $LD_{50/30}$'s than continuous exposures of the same duration. However, results from 4-fraction and 18-fraction experiments were not appreciably different from those of comparable continuous exposures. Worthy of note is that in the 18-fraction experiment, the interval between the i^{th} and $i + 1^{st}$ exposure ($i = 1, 2, \dots, 17$) was considerably less than 6 hr. In the 4-fraction experiment, the intervenient time between fractions varied from about 1.5 to 6 hr. Acute mortality data from recent experiments in which LAF₁ mice were exposed to Co^{60} γ -rays confirm the observations of Brown et al.⁽⁶⁾ for split and protracted exposures (see below). However, these differences between effects of split and protracted exposures can be explained by extending the model to include among its basic assumptions that the reversibility of time-dependent effects is limited in time.

How does the assumption of limited reversibility explain the experimental observation that, with split doses separated by roughly 6 or more hr, reduction in dose-effectiveness seems to have stopped; whereas with protracted exposures, reduction seems to continue for protractions of considerably longer times? Under such an assumption is it to be expected that the results with fractionated exposures could be in some cases indistinguishable from those under protracted exposure; in other cases, indistinguishable from those with split exposures; and in still other cases, could the pattern of mortality fall between those for the other types of exposure? For greater clarity the limited reversibility assumption is stated as follows:

There exists a time T , a constant, such that time-dependent effects produced during exposure between time t and $t + \Delta t$ ($\Delta t > 0$), and not reversed prior to or within the interval $t + T$ and $t + \Delta t + T$, can no longer be reversed.

Application of Model to Split and Fractionated Exposures

If a single protracted exposure is given, a reduction of reversible effects takes place at a constant rate B during the entire exposure* since at each instance during exposure, radiation effects exist that are capable of being reversed. For split or fractionated exposures with maximum inter-venient time less than T , dose reduction at rate B also takes place through the end of the last exposure given. In this case the dependence of per cent mortality on dose is the same as with protracted exposures. But if an inter-venient time exceeds T , say by C units of time, no effects are reversed during the last C units of the interval. Consequently, the effect that remains at the end of the interval is greater than expected if reversal had continued for the entire interval. Figure 5 diagrams the accumulation of reversible effects after protracted and split exposures for a hypothetical mouse i with

Figure 5

Hypothetical example of injury accumulation under protracted and split exposures. Symbols are defined in text. Solid line, continuous exposure; dashed line, split exposure.

* We consider only the case in which the rate of production of effects (dose rate) is greater than the reversal rate.

reversal rate B_i and lethal bound Y_{0i} . For the protracted exposure, effects are produced uniformly at a constant rate R from time zero to $T + C$. The gross effects produced in this interval equal $2Y$ (equivalent to a total dose of $2D$). For the split exposure, two equal effects, each of magnitude Y and each equivalent to a dose D , are produced instantaneously at time zero and at time $T + C$. It is seen (Figure 5) that at time $T + C$ (the instant after delivery of the second exposure), the effects remaining from the split exposure exceed by $B_i C$ the residual effect for the single protracted exposure even though the total effects produced were the same. In this example, residual effects from the split exposures exceed the lethal bound Y_{0i} ; thus mouse i will die as a result of this injury. Under continuous exposure, mouse i will survive these effects. It is assumed, for this illustration, that the lethal bound associated with mouse i for the irreversible effect is not exceeded by the total irreversible effect produced by a dose of $2D$. Incidentally, at times exceeding $2T + C$, reversal should have completely stopped in both cases.

Application of Model to Experiment

Description of the kinetics of injury accumulation in the mouse in terms of experimentally controlled or observable quantities has been achieved under this model for protracted exposures with doses within the 30-day lethal range.⁽⁵⁾ Estimates of only three characteristics of the mouse population under consideration are needed: 1) the time constant τ ,* 2) the distribution of sensitivities to the reversible effects, and 3) the distribution of sensitivities to irreversible effects. These estimates have been determined for the LAF_1 mouse exposed to Co^{60} γ -rays, it was also demonstrated that other strains of mice continuously exposed to different qualities of ionizing radiations express similar injury kinetics.⁽⁵⁾ The principal differences between and within strains can be attributed to variations in magnitude of the instantaneously delivered dose that is required for unit effect.

In a series of split-dose exposures, the LAF_1 mouse was again used. Groups averaging 50 mice each were given a conditioning dose of 700 r from a Co^{60} source.⁽⁷⁾ Both sexes were included. A second dose was given at various intervals from 0 to 1440 min, inclusive, after the first exposure. For each interval, groups received a variety of doses that were chosen so as to bracket the median lethal dose for mortality between 9 and 30 days. Other experimental conditions were similar to those previously reported for protracted exposures.⁽⁵⁾ By probit least squares, the LD_{50} and associated standard error were estimated (Table 6). Figure 6 gives the ratios of D , the LD_{50} at each interval greater than zero, to D_0 , the LD_{50} at the zero interval, or single-dose exposure. The theoretical curves for protracted and split exposures are based on constants estimated from experiments using protracted exposures.⁽⁵⁾ The estimate of τ is 1419 min; the LD_{50} and D_0 's for the reversible effects are 969 r and 82 r, respectively; and the corresponding values for the irreversible effects are 1389 r and 155 r,

---

* The symbol τ represents the time constant associated with the time-dependent injury process (see statement of model, above).

respectively. Brown et al.⁽⁶⁾ suggested that the departure in mortality pattern between split and protracted exposures occurs at about 6 hr; we have seen it as early as 4 hr. Thus, we chose $T = 5$ hours, the mean of these observations.

TABLE 6
Probit least squares constants of the distribution of mortality from 9 to 30 days after beginning of exposure (split dose, with a first dose of 700 r)

Intervient interval, ° min	LD ₅₀ , r	Standard error of LD ₅₀ , r	Slope of probit line	Standard error of slope**	D/D ₀	Intervient interval, ° min	LD ₅₀ , r	Standard error of LD ₅₀ , r	Slope of probit line	Standard error of slope**	D/D ₀
0	989*	6	0.01514	0.00176	1.	360	1178	9	0.00811	0.00078	1.191
60	1063	14	0.01132	0.00301	1.075	480	1172	13	0.01031	0.00166	1.185
120	1123	10	0.00983	0.00148	1.135	960	1232	29	0.00564	0.00154	1.246
240	1195	8	0.01079	0.00124	1.208	1440	1194	32	0.00609	0.00210	1.207

* In addition, 20 min was required to deliver the two exposures.

** The estimate of the standard deviation of the distribution is equal to the reciprocal of the slope.

* D₀.

Symbol	Exposure pattern	Radiation	D ₀ , r	Strain	Reference
•	Split doses, 700 r first dose	Co ⁶⁰ γ-rays	989	LAF ₁	This report
Δ	Split doses, equal fractions	250 kvp X-rays	792	R	(6)
×	Split doses, 400 r first dose	250 kvp X-rays	883	CBA	(8)
○	Split doses, 400 r first dose	250 kvp X-rays	578	CF No. 1	(9)
▼	Protracted exposure	250 kvp X-rays	792	R	(6)
+	Protracted exposure	Co ⁶⁰ γ-rays	969	LAF ₁	(5)

Figure 6. D/D_0 ratio of median lethal doses versus exposure or intervenient time. Solid line, theoretical curve for protracted exposures; dashed line, theoretical curve for split exposures.

As was demonstrated with protracted exposures,⁽⁵⁾ very good agreement exists between our results with split exposures and similar findings of other investigators (Figure 6). It thus seems that use of the normalized variable, D/D_0 , will greatly facilitate comparisons of mechanisms of acute radiation injury between and within strains and species.

References

1. Tyler, S. A., and S. P. Stearner. Discrimination among injury processes reflected in acute radiation mortality. Intern. J. Radiation Biol. 4, 495-509 (1962).
2. Stearner, S. P., and S. A. Tyler. An analysis of the role of dose and dosage rate in the early radiation mortality of the chick. Radiation Res. 7, 253-266 (1957).
3. Tyler, S. A., and S. P. Stearner. Modes of radiation death in the chick embryo. II. A model of lethal mechanisms. Radiation Res. 12, 301-316 (1960).
4. Stearner, S. P., and S. A. Tyler. Comparative aspects of acute radiation mortality in birds. Intern. J. Radiation Biol. 5, 205-215 (1962).
5. Stearner, S. P., and S. A. Tyler. Radiation mortality in the mouse. Model of the kinetics of injury accumulation. I. Protracted doses in the 30-day lethal range. Radiation Res., in press.
6. Brown, J. A. H., M. J. Corp, and R. H. Mole. The effect of dose-rate and fractionation on acute mortality in X-irradiated mice. Part II. Intern. J. Radiation Biol. 5, 369-377 (1962).
7. Tyler, S. A., and S. P. Stearner. Accumulation of acute injury in the mouse subjected to split or fractionated doses of X- or gamma radiation. Radiation Res., in press.
8. Mole, R. H. Quantitative observations on recovery from whole body irradiation in mice. Brit. J. Radiol. 29, 563-569 (1956).
9. Storer, J. B. Rate of repair of radiation damage in mice. Atomic Energy Commission Conference on the Shorter-Term Biological Hazards of a Fallout Field, (ed. G. Dunning) U. S. Gov. Printing Office, Washington, D. C., 1956.

THE TOXICITY OF Ra^{226} IN MICE: A PROGRESS REPORT

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

In May, 1960, we began a second experiment with mice exposed to Ra^{226} . The data from some dose-level groups are now complete, and from the others they are available through 500 days after injection. This seems a good interval at which to compare these data with those from the first Ra^{226} toxicity experiment.⁽¹⁻⁴⁾ The original experiment is being repeated because it was seriously troubled with infectious disease and because its dose-response curve for the induction of osteogenic sarcomas is quite unlike that of any other bone-seeking radioisotope.

Materials and Methods

Female CF1/An1 mice were given an intravenous injection of Ra^{226} bromide when they were about 70 days old. They were housed initially 15 per cage in stainless steel cages having wire mesh floors. During the course of the experiment sick, debilitated, or pugnacious mice were isolated in plastic cages with wood shavings as bedding, where they remained until death.

The original plan was only to examine the skeleton roentgenographically after death and score the malignant bone tumors. Bones with questionable lesions on the basis of X-ray appearance were to be examined histologically. It soon became apparent that very little effort was saved by omitting gross autopsy, and very much information was being lost. So, after the experiment had been under way for a few weeks, gross autopsy was added to the routine procedures.

The 2250 mice listed in Table 7 entered the experiment in 9 installments over a period of 3 months. The animals of each installment were distributed proportionately among the 12 dose-level groups. The fourth installment, which consisted of 465 mice, was discarded within a month of injection because ectromelia was suspected in some cages and an intestinal infection in others. These animals were replaced later, along with another two cages of control mice that had been located on the same rack.

Even though all the mice had been vaccinated against ectromelia soon after weaning, they were revaccinated at this time and again 11 months later. Very likely the first vaccine used had been inactive. Other medication given uniformly to all mice alive at the time has included treatment for ectoparasites consisting of two Aramite baths given 8 months after the start of the experiment,⁽⁵⁾ and injections with Terramycin, which were given at 21 months in an attempt to curb the epidemic described below.

TABLE 7
Current results

Ra ²²⁶ injected, $\mu\text{C/kg}$	No. of mice	Survival		Osteogenic sarcomas				Reticular tumors	
		Life expectancy, days	Survival at 500 days, %	Av. tumor expectancy per mouse after 250 days	Per cent with tumors at 500 days	Tumors per mouse at 500 days	Day of first tumor death	Day of maximum mortality rate	Maximum daily rate/ 10,000
120	45	287.9	0	1.11	31.1	0.47	260	285	15.4
80	45	349.8	2.2	1.89	66.7	1.27	276	265	14.6
40	45	380.3	8.9	1.73	62.2	1.16	286	265	19.6
20	45	425.8	17.8	1.84	66.7	1.02	274	375	16.2
10	45		48.9		33.3	0.38	295	365	16.6
5	45		75.6		13.3	0.16	365	395	20.0
2.5	105		69.5		4.8	0.05	354	305	13.8
1.25	105		84.3		1.0	0.01	392	465	8.6
1.0	240		80.4		2.1	0.02	465	475	7.6
0.75	510		82.0		1.0	0.01	344	435	6.4
0.5	600		80.6		1.7	0.02	380	425	9.6
0	420		82.1		0	0	-	465	6.0

At time of injection

Results

Survival. Except for the initial difficulty with the fourth injection installment, the mice remained remarkably free of infectious disease for 20 months. Six hundred days after the first injection, daily mortality from all causes for the total population was only 2/1000 (Figure 7). However, there was a sudden change, and 30 days later daily mortality was 13/1000. A subsequent decline brought it to 3.5/1000 at 670 days, but during these two months more than 10% of the living population had died with an acute disease characterized by failure to eat and congestion of the lungs.⁽⁶⁾ The disease has not occurred again in epidemic proportions, but from that time on normal lungs rarely have been seen at autopsy.

Figure 7

Mortality rate per 100 mice calculated at 10-day intervals for mice of all dose-level groups combined.

The survival data of the four highest dosage groups were not influenced by the epidemic because the last of these mice had died by 610 days after injection. Their life expectancies at the time of exposure to radium are given in Table 7.

At 500 days the mortality of mice that had received $2.5 \mu\text{c/kg}$ or more was greater than that of mice that had received $1.25 \mu\text{c/kg}$ or less (Table 7). Among this latter segment of the experiment there was no consistent difference between the control mice and those that had received radium. The mortality rate values also indicated that the dividing line between effective and ineffective levels during the first 500 days lay between 2.5 and $1.25 \mu\text{c/kg}$.

Osteogenic sarcomas. The first death with an osteogenic sarcoma occurred at 260 days after injection, and from that time on almost every mouse that had received $20 \mu\text{c Ra}^{226}/\text{kg}$ or more died with at least one malignant bone tumor. The average number of tumors expected among these animals from 250 days through the time during which at least 3 remained alive varied from 1.1 to 1.9 (Table 7). Eighty, 40, and $20 \mu\text{c/kg}$ were equally carcinogenic, but $120 \mu\text{c/kg}$ was less potent.

Incidence data at 500 days are given both as percentage of animals dying with tumors and as average number of tumors per mouse at death. The latter values are more meaningful because they include all the osteogenic sarcomas, those that were one of several in an animal as well as those that appeared alone. The carcinogenic response decreased as the amount injected decreased from 80 to $1.25 \mu\text{c/kg}$, but the trend was lost when the amount injected was further decreased to $0.5 \mu\text{c/kg}$. No osteogenic sarcomas had appeared among the 420 control mice by 500 days. However, in previous experiments the incidence in control CF No. 1 female mice has been 1-2%^(1, 4)

The time of first death with an osteogenic sarcoma in each dose-level group is given in Table 7. Whenever we examine data of this kind we are impressed with the exceptions to any supposed pattern of time-dosage relationship. Rather than suggesting that the latent period to tumor death increases as the dosage decreases, these data suggest that the latent period is independent of the amount of radium that had been injected. When tumor pressure is so great that the daily mortality rate with tumor is very high, the probability of a death with tumor immediately after the latent period also is very high. When tumor pressure is low, the probability of a death with tumor immediately after the latent period also is low, and the time of the first tumor death would be expected to vary considerably. The evidence in support of this hypothesis is that the first tumor death occurred between 260 and 286 days in those groups in which the average mouse had more than one tumor by 500 days, but it ranged from 344 to 465 days when there were only 1-2 tumors among 100 mice.

Reticular tissue tumors. When gross autopsy was made part of the routine procedures, data on tumors of the blood-forming tissues became available. However, since in the CF No. 1 mouse precise classification is not possible without histologic examination, this family of tumors is being treated here as a single neoplastic disease. This practice is not entirely satisfactory because there is evidence that the specific tumor types respond differently to radiation,⁽³⁾ but it is not without some value.

Among the control mice daily mortality with reticular tumors increased gradually from less than 1/10,000 at about 200 days after injection to about 5/10,000 at 300 days and 6/10,000 at 465 days. None of the groups that had received 1.25 μc Ra^{226} per kg or less was very different from the controls; however, each had a maximum rate some time between 400 and 500 days that exceeded the control maximum (Table 7). At the higher dosage levels the tumors appeared sooner and in greater number.

Discussion

In the first standard Ra^{226} -toxicity experiment life span was not significantly changed by 1.2 $\mu\text{c}/\text{kg}$, but the data from the groups receiving 3 through 28.8 $\mu\text{c}/\text{kg}$ were so variable that conclusions could not be drawn. In the present experiment 2.5 $\mu\text{c}/\text{kg}$, but not 1.25 $\mu\text{c}/\text{kg}$, had an effect upon survival by 500 days. It is hoped that the subsequent data will not lose their reliability because of the epidemic experienced after the experiment had been in progress for 600 days.

The incidence of osteogenic sarcomas in the first experiment was increased by 1.2 $\mu\text{c}/\text{kg}$ but not by 0.6 $\mu\text{c}/\text{kg}$. Current results put 0.5 $\mu\text{c}/\text{kg}$ within the carcinogenic range. Thirteen tumors had appeared among the 600 mice in this group by 500 days, and an additional 31 already have been tabulated between 501 and 880 days. Consequently, the final incidence will be at least 7.3%. This value is unexpectedly high, particularly since only one osteogenic sarcoma has been counted to date among the 420 control mice.

The very low incidence of malignant bone tumors in the control mice is equally unexpected. In the past, 1-2% of untreated CF No. 1 female mice have died with osteogenic sarcomas. No obvious changes have been made in the physical situation of the animals that might account for the difference. The cages are the same, and, as far as we can judge, the food, water, temperature, humidity, and general care are the same. However, the animals themselves may be quite different. In almost all previous experiments the mice have been supplied by Carworth Farms, arriving in the Laboratory when they were 5-6 weeks old. The present animals were bred in the Laboratory from a stock established a year or so earlier. The first mice of the new colony consisted of only a few litters delivered by cesarean section.

A shift in gene frequency is not uncommon after a sharp reduction in the size of a breeding population if the population is not inbred. Since random breeding was the usual procedure at Carworth Farms, such a shift could account for the change in the incidence of malignant bone tumors. We also have the impression that the incidence of mammary and lung tumors is lower in the CF1/An1 mice, and that fewer cases of thymic lymphoma early in life have been seen.

The high incidence of osteogenic sarcomas in the mice that received the smallest amount of Ra^{226} means that the carcinogenicity of even smaller amounts must be investigated. Consequently, the experiment is being extended to include another 2250 mice. Amounts being injected are 0.5, 0.25, 0.1, 0.05, and 0.0 $\mu\text{c/kg}$.

References

1. Finkel, M. P. Relative biological effectiveness of radium and other alpha emitters in CF No. 1 female mice. *Proc. Soc. Exptl. Biol. Med.* 83, 494-498 (1953).
2. Finkel, M. P. Relative biological effectiveness of internal emitters. *Radiology* 67, 665-672 (1956).
3. Finkel, M. P. Late effects of internally deposited radioisotopes in laboratory animals. *Radiation Research Suppl.* 1, 265-279 (1959).
4. Finkel, M. P. and B. O. Biskis. The induction of malignant bone tumors in mice by radioisotopes. *Acta Union Intern. Contra Cancer* 15, 99-106 (1959).
5. Flynn, R. J. Control of ectoparasites in mice. *Proc. Animal Care Panel* 6, 75-91 (1955).
6. Flynn, R. J., I. L. Greco, and P. B. Jenkins. A disease of mice characterized by lung congestion. *Lab. Animal Care* 13 (4), Part 1, 499-501 (August 1963).

TOXICITY AND METABOLISM OF RADIONUCLIDES IV. PROGRESS REPORT FOR PERIOD ENDING DECEMBER 31, 1962

William P. Norris, Louise S. Lombard,
Carl E. Rehfeld and Thomas W. Speckman

This is the fourth semiannual report of studies on the metabolism and toxicity of fission products in pure bred beagle dogs. Major attention was given the question of whether the radiation sensitivity of the beagle, as judged by its response to single, near-lethal doses of Cs^{137} , is appreciably dependent upon the age of the animal. The results of this study and certain others, largely related to age effects, are reported here.

Toxicity of Cs^{137}

Fifty-three dogs in all have been given intravenous injections of Cs^{137} in the acutely lethal range, and 49 of these have been observed for survival. Age at injection, dose, and survival are presented in Table 8.

TABLE 8

Survival of beagles following a single intravenous dose of Cs^{137}

Dog No.	Sex	Date of injection	Age at injection, days	Amount injected		Days to death	Survival at 12-31-62, days	Dog No.	Sex	Date of injection	Age at injection, days	Amount injected		Days to death	Survival at 12-31-62, days
				mc	mc/kg							mc	mc/kg		
397	M	4-17-62	151	35.29	3.67	-	258	313	F	11-06-62	561	27.73	3.18	Sacrificed for pathology	
398	M	"	151	32.27	3.79	27		316	F	"	561	28.34	3.14	"	
399	M	"	151	30.26	3.24	-	258	366	F	"	399	24.26	3.17	-	55
401	F	"	151	29.40	3.68	27		367	M	"	399	25.91	3.09	36	
404	F	"	142	39.52	3.65	-	258	369	M	"	399	24.52	3.10	28	
470	M	10-23-62	151	25.22	3.85	-	69	390	F	12-05-62	395	29.27	3.31	-	26
471	M	"	151	21.91	3.76	-	69	391	F	"	395	28.03	3.34	-	26
472	F	"	151	17.19	3.65	-	69	392	F	"	395	26.77	3.45	-	26
473	F	"	151	21.22	3.79	-	69	393	F	"	395	23.85	3.41	-	26
474	F	"	151	23.80	3.60	-	69								
485	M	10-31-62	149	27.50	3.64	-	61	107	M	2-16-61	ca 1023	26.32	1.99	64	
486	M	"	149	24.57	3.59	-	61	121	M	"	ca 1023	26.09	2.04	27	
487	M	"	149	25.79	3.70	39		124	M	"	ca 870	20.40	1.58	-	683
491	F	"	148	20.09	3.65	-	61			10-02-61		16.15	1.04		
492	F	"	148	22.10	3.76	-	61	114	M	10-31-61	ca 731	7.50	.93	-	791
										8-14-61		10.76	1.01		
52	M	4-11-61	415	17.56	2.11	Accidental death				4-10-62		11.63	1.13		
53	M	"	415	22.62	2.11	"									
54	M	"	397	17.05	1.98	-	629	128	M	10-31-60	ca 762	11.75	1.08	-	791
55	M	"	397	17.17	2.11	-	629			8-14-61		11.89	0.99		
81	M	"	397	15.52	1.85	-	629			4-10-62		12.83	1.01		
246	F	9-19-61	398	22.52	2.43	-	468	46	F	9-25-62	1387	36.65	3.74	21	
247	F	"	398	21.86	2.16	-	468	48	F	"	1509	33.60	3.64	20	
256	F	"	404	18.50	2.27	-	468	69	M	"	1662	42.45	3.71	24	
257	F	"	404	20.20	2.23	-	468	71	M	"	1662	30.49	3.70	22	
258	F	"	404	24.66	2.32	-	468	72	M	"	1662	34.60	3.79	22	
237	F	1-18-62	417	31.91	3.59	28		60	F	11-20-62	2066	23.11	3.39	24	
239	M	"	417	34.44	3.36	-	347	63	F	"	2060	25.23	3.32	26	
241	F	"	417	26.48	3.69	31		64	M	"	2060	19.75	3.29	29	
243	M	"	417	40.22	3.33	35		65	M	"	2060	24.43	3.40	28	
								80	M	"	2060	26.16	3.27	24	

These groups of dogs are discussed in the section on Hematological Effects

Living at the time of reporting, unless otherwise noted.

The dependence of radiosensitivity on age was investigated in dogs of 3 somewhat arbitrarily chosen ages: 5 months, 13 months, and 4-5 years. The animals were injected in groups of 4 or 5 at a time. Since in 13-month-old beagles, the 30-day LD₅₀ was close to 3.5 mc Cs¹³⁷/kg, this dose was used to test the response of dogs in the other 2 age groups. Animals were injected at different times and the actual dosages delivered varied slightly from group to group.

Typically, all dogs passed through an acute period between 25 and 30 days after injection which was associated with hematopoietic damage. Most of the deaths occurred during this time. In surviving animals, attempts at regeneration were observed shortly after 30 days, and few deaths occurred much later than this.

There seems to be little doubt that the radiosensitivity of the beagle varies appreciably even during the first year or two of life. Of the 15 5-month-old beagles given an average of 3.7 mc/kg, only 3 died. However, of the 11 13-month-old dogs given 3.3 mc/kg, 5 died; the fact that 4 of the survivors were litter mates poses certain obvious questions regarding lack of uniformity of experimental animals. There were no survivors among the 10 dogs 4-5 years old that were given comparable doses; in fact, the earlier death of these animals further emphasizes their inability to withstand the treatment.

Hematological Effects of Cs¹³⁷*

Clinical hematology is one feature of the over-all study of the dogs included in Table 8, but discussion here is limited to a portion of the results obtained from the three representative groups indicated.

These dogs receive routine care and are given daily clinical examinations to detect any effects due to the presence of Cs¹³⁷ retained in the body but are not given medical treatment for any such conditions. They are either anesthetized or tranquilized during the injection of Cs¹³⁷; this procedure also facilitates the whole-body radioactivity measurements made immediately thereafter. Additional whole-body measurements are usually made but without the aid of drugs. Blood samples are drawn periodically from most of the dogs.

Three weekly blood samples are taken from each dog before the injection of Cs¹³⁷, and additional samples are taken once or twice a week thereafter until death or until obvious recovery. When the cellular elements have increased and attained a fairly stable level, sampling is done at 1-month to 6-month intervals. Initially the cells were counted by hemocytometry, but most of the counts have been made with an electronic

---

*This work was done with the technical assistance of Donald E. Doyle.

particle counter (Coulter). During the transition from one method to the other numerous duplicate counts were done, resulting in the conclusion that the electronic method is the more efficient.

The observations reported here include leukocyte and erythrocyte counts (number/mm³ of whole blood), hemoglobin (g/100 ml), hematocrit (% packed red cells), and erythrocyte sedimentation rate. Mean cell volume (MCV) and mean corpuscular hemoglobin concentration (MCHC) were calculated according to the Wintrobe method. Leukocyte differentiation has been completed for only one group of dogs.

Control means for each blood value were calculated from the data obtained from the 3 pre-injection samples. The control means varied significantly from group to group and, also, the various groups were placed on experiment at different times as they reached the proper age. Therefore, the data from each group of dogs are pooled but graphed separately from other groups.

The results from the first group of 150-day-old dogs are summarized graphically in Figures 8A and 8B. The blood samples were obtained at about weekly intervals in this group. Each point is the average of results obtained on all 5 dogs until the 27th day and for 3 dogs thereafter. The values became fairly stable after 70 to 80 days, so results are not shown for the entire survival period.

The leukocyte count decreased from an average of over 15,000 per mm³ to approximately 5000 within the first few days and dropped to a low point of 1000 or less between 20 and 30 days after injection. Recovery was gradual but never complete to the pre-exposure level although a leukocyte value of 10,000 is considered normal for healthy control dogs of the same age.

Erythrocytes are decreased. It is probable that production is depressed, but the effect is not immediately apparent because erythrocytes have a longer life span than do leukocytes. Anemia is apparent; it is marked in the nonsurvivors and persists for some time in the convalescent dogs.

In 5-month-old dogs it appears that mean cell volume (MCV) is increased and that MCH keeps pace with this increase in cell size (Figure 8B). Although MCHC appears to be rather erratic in this group of dogs it is actually rather stable, in that it deviates no more than 11% from the control mean.

The data from the second and third groups of 150-day-old dogs (figures not shown) support those from the first group. In the second group there were no deaths and no abnormal increases in the sedimentation rate of the erythrocytes. If the calculated values for hemoglobin concentration for all three groups are included in one curve the variation from the control values is not significant.

Figure 8 Values obtained from samples of peripheral blood of dogs given 3.5 mc of Cs^{137} per kg of body weight at 150 days of age. Averages were taken from 5 dogs to 27 days (arrow) and from 3 dogs thereafter A, leukocytes (WBC), erythrocytes (RBC), hemoglobin (HGB), hematocrit (HCT), sedimentation rate (SED). B, mean corpuscular hemoglobin concentration (MCHC), mean corpuscular hemoglobin (MCH), mean cell volume (MCV).

Four dogs were given 3500 $\mu\text{c/kg}$ Cs^{137} at 417 days of age (Figure 9). Three of these dogs died after having Cs^{137} for 28, 31, and 35 days, hence the values are averages of values obtained from four dogs up to 28 days, from two dogs between 28 and 35 days, and for the single survivor after 35 days.

From the data from the dogs injected at 417 days of age it is apparent that the leukocytes in the peripheral blood were reduced in number by 5000 within the first 4 days. Thereafter the reduction was more gradual but the average count was less than 1000 cells/mm³ of whole blood for at least a week. Leukogenesis in the one surviving dog (239) recovered to the point where there were approximately half as many leukocytes at 135 days as there were before injection of the isotope.

Figure 9. Values obtained from samples of peripheral blood of dogs given 3500 μ c of Cs^{137} per kg body weight at 117 days of age. Averages taken from 4 dogs to 28 days, 2 dogs from 28 to 35 days, and one dog to 135 days (times indicated by arrows)

tion (MCHC) seem to indicate that average cell size may be increased and the hemoglobin concentration as well (Figure 10).

Differential leukocyte counts were made using blood films stained with Wright's stain. Control means and values indicated by the curves represent total numbers or absolute values for each type of leukocyte per mm³ of whole blood (Figure 11).

The agranulocytes were not depressed completely during the critical period 25 to 35 days after injection. Lymphocytes were affected most within the first four days as shown by the decrease from a mean of 4854 to 1200 in that interval of time. Aberrant forms were seen during the critical period of all the dogs and in the recovery period of one dog.

Erythrocyte numbers were maintained very well in the dogs of this group for the first 2 weeks after injection but declined rapidly thereafter to a value of half the control mean between 28 and 40 days. The initial slight increase seems best explained as due to a decrease in blood plasma caused by dehydration. No efforts have been made to ascertain exact differences in fluid intake or loss in any of the animals although intake was estimated to be normal or greater than normal; the volume of urine appeared to be increased.

Hemoglobin and hematocrit values correspond well with the variation in number of erythrocytes for the initial period of about 30 days. These values returned to normal within 135 days in the surviving dog, but the number of erythrocytes remained less than the pre-exposure level. Sedimentation rate was maximal in the dogs that died but in the surviving dog it did not reach such a critical point until after there were indications of restored leukogenesis. The calculated values for mean erythrocyte volume (MCV), hemoglobin (MCH), and hemoglobin concentra-

Figure 11

Differential leucocyte counts (per mm^3 of peripheral blood) of dogs given $3500 \mu c$ of Cs^{137} per kg body weight at 417 days of age. Averages taken from 4 dogs to 28 days, 2 dogs from 28 to 35 days, and one dog to 135 days (times indicated by arrows).

Of the granulocytes, the neutrophils were markedly reduced in number with a steady reduction to almost complete disappearance at 30 to 35 days after injection. During the period of extreme depression, the staining and the morphological characteristics were definitely abnormal but typical of those often associated with extreme debility and toxicity.

Eosinophils persisted in the normal number for at least 10 days, hence there was relative but not actual eosinophilia. Basophils were also somewhat more resistant to the effects of radiation during the first few days of exposure, if persistence in circulating blood is a criterion of resistance.

Hematology data from the second and third groups of dogs given Cs^{137} at approximately 13 months are not included in this report, but a preliminary survey suggests that they will support the results from the first group.

Severe effects upon the hemopoietic system were apparent in the 5 dogs that were given 3500 $\mu\text{c/kg}$ at about 1576 days of age. At 4 days there were 40% fewer leukocytes in the peripheral blood, and, although the rate of decrease was less thereafter, the counts at the time of death ranged from 1500 to less than 1000 leukocytes per mm^3 of whole blood (Figure 12).

Figure 12

Values obtained from samples of peripheral blood of dogs given 3500 μc of Cs^{137} per kg body weight at 1567 days of age. Averages taken from 5 dogs through 15 days and 4 dogs through 18 days (arrows indicate times).

Erythrocytes, hemoglobin content, and hematocrit of the blood all decreased, and the degree of anemia was no doubt more severe than it appears to be as shown graphically (Figure 12). These dogs appeared to be mildly to severely dehydrated during most of their survival period. The sedimentation rate did not increase significantly before death in one of the dogs and never to the same rate in the others as it did in the young dogs. The calculated values for corpuscular volume, hemoglobin, and hemoglobin concentration do not suggest important changes. There was apparently insufficient time before death for effects on erythropoiesis to be manifested.

Some observations and conclusions are worth noting concerning the effects of Cs^{137} when given at a dose level of $3500 \mu\text{c}/\text{kg}$. Leukopenia and anemia are characteristic. When leukopenia, erythropenia, and a high sedimentation rate occur simultaneously, the prognosis is unfavorable. If there is evidence of vigorous leukogenesis and the sedimentation rate is not too high, the prognosis is more favorable and it does not matter greatly that the dogs become increasingly anemic for a time. Anemia is not the cause of death although it probably is contributory. A prolonged maturation time and delayed release of the cells into the circulatory system would contribute to an erythropenia. It is quite likely that the bone marrow would appear hyperactive. Water intake is maintained but urine volume probably is increased because dehydration is usually obvious. Lymphocytes persist in the peripheral blood but they are markedly decreased and their appearing in a higher percentage is only a relative increase. Eosinophils, in particular, and basophils, to some degree, persist in the peripheral blood longer than the neutrophils, but all granulocytes may be practically non-apparent during the period when there is most evidence of toxicity.

Certain differences are noted in the reaction to Cs^{137} toxicity when the three age groups are compared. The severe leukopenia occurred at different times in relation to the time of death: It occurred in the 150-day-old dogs in the survivors after the others had died, and in the 400-day-old dogs about one week prior to and during the time that deaths occurred; in the older dogs the initial point of severe depression occurred at the time of death.

From these initial observations it appears that recovery of leukogenesis varies with age. Survivors that were injected at 150 days of age were found at 130 days after injection to have normal peripheral leukocyte counts for their age. Complete recovery has not occurred in the 400-day-old dogs. There were no survivors in the older age groups, and death occurred essentially at the point of initial severe leukopenia.

Anemia was of about equal severity in the 150- and the 400-day-old dogs, but it occurred earlier in the young mature dogs, and recovery was much better in the immature. Red cell counts did not indicate severe

anemia in the 1500-day-old dogs in their short period of survival, but it is possible that the effect was sufficient to contribute as a cause of death. In general the anemias were not sufficient in themselves to cause death in any of the three age groups

Variation in Total Potassium Concentration with Age and Sex in Beagles*

These investigations of potassium were initiated as a result of our interest in the metabolism and toxicity of cesium in beagles. Potassium is chemically similar to cesium, both being located in periodic group Ia, comprising the alkali metal elements. The two elements have been considered similar as to biochemical behavior. Naturally occurring potassium contains a naturally radioactive nuclide (K^{40}) with a per cent abundance of 1.19×10^{-2} and physical half-life of 1.25×10^9 years. The 1.46-MeV gamma radiation arises from electron capture and occurs in 11% of the disintegrations.⁽¹⁾ This amount makes it possible to determine the total potassium content of an animal in vivo by measuring the gamma radiation from K^{40} . This experiment was planned to ascertain whether total potassium concentration varies significantly with age and sex of the beagle.

The beagles used in this experiment were from our stock colony. No dogs had been subjected to any experimental work, and no breeding bitches were used. The beagles had all been fed only Rockland Dog Food (A E Staley Co, Decatur, Illinois) which contains 0.84% potassium. The dogs were all housed in combination inside-outside runs approximately 3 by 12 feet in which they had complete freedom of movement. The variation in animal age was obtained by sampling the existing dog colony.

Gamma radiation measurements were performed inside a 4 x 4 x 4-ft steel room constructed from single 6-in. steel plates. The detector was a 4 x 8-in. NaI(Tl) crystal coupled to a 5-in. EMI photomultiplier tube. The signal was analyzed with a 400-channel pulse-height analyzer (Radiation Instrument Development Laboratory). The dogs were restrained in an arc around the detector (Figure 13). The radius of the back plate of the arc was 14 in. and the midsagittal plane of the dog was always placed 11 in. from the center of the detector. The midsagittal plane was determined by averaging the measured thicknesses at the umbilicus and xiphoid cartilage. A tranquilizer, Promazine hydrochloride, was administered to calm the dogs for the 45-min counting period: half the dose was given intravenously and half intramuscularly. The measurements were made during the normal work day, with a 15-hr background measurement during the preceding night and a 15-hr standard measurement during the subsequent night.

---

*This work was done with the assistance of W. A. Hendrickson (Summer Student Aide) and N. D. Kretz.

Figure 13. Position of dog for counting low-level gamma-emitting isotopes.

The standard was potassium iodide prepared from analytical reagent grade potassium iodide weighed in amounts sufficient to prepare three standards. These were compared by gamma measurements and one was used as the standard throughout the experiment. The correction factor between the standard and the dog was determined by comparing syringes containing K^{42} (1.53 MeV gamma) with the standard in its usual position of measurement, and by measurement of the same K^{42} following intravenous injection into dogs of various sizes and measured in the standard position. Five beagles ranging in weight from 5 kg to 11 kg were used for this determination. The correction factor so obtained was plotted versus weight and applied to all dog measurements. Data are expressed as milliequivalents of potassium per kilogram body weight.

The results of measurements of 48 female and 56 male beagles are shown in Figure 14. Individual measurements vary considerably, presumably reflecting the cumulative effects of individual variation, normal difficulties experienced in low-level counting, and possibly errors in placement of the beagle for counting. However, values from all animals of a given age are averaged, the points present the indicated curves.

(A)

(B)

Figure 14. Variation in total-body potassium concentration with age in female (A) and male (B) beagles.

When viewed without data points (Figure 15), the curves closely parallel those of Anderson and Clinton⁽²⁾ describing potassium concentration in humans, and also they are qualitatively the same. This is in some disagreement with the results of Richmond and Furchner⁽³⁾ describing potassium concentration in beagles as being approximately 20% greater than humans.

Figure 15

Variation in total-body potassium concentration with age and sex in beagles.

Our results also show a definite change in potassium concentration with age (Figure 15). It may be pointed out that in some instances in which several points are either higher or lower than the average it was found that the animals involved were litter mates; this may indicate hereditary differences reflected in the general build of the dog.

Further studies are planned to firm up portions of the curve. A pneumatic device for the in vivo determination of body volume is being constructed, and it is hoped that utilization of the density of the animal at time of measurement will aid in interpretation of results.

Effect of Co⁶⁰ Gamma Irradiation upon Urinary Constituents of Beagle Dogs*

The dogs listed in Table 9 were subjected to Co⁶⁰ γ -irradiation in the high-level gamma room in an attempt to duplicate a previous experiment in which dogs 0147 and 0151, after unilateral γ -irradiation with 325 r, had excreted a reducing substance for a period of 150 days. Irradiation was unilateral to the left side.

---

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

TABLE 9

Experimental plan for irradiation

Dog No.	Age at irradiation, days	Sex	Total dose, r	Survival, days after exposure
147	770*	M	325	196
151	770*	F	325	196
302	405	F	325	217
303	398	F	704	15**
422	275	F	325	35
424	275	M	325	35

*Second irradiation.

**Time to death. The other five dogs are still alive at the time of reporting.

Sixteen-hour (147, 151, 302, 303) and 24-hour (422, 424) urine samples were collected for periods up to 52 days after irradiation. None of the above animals, including 147 and 151 which were irradiated for the second time, excreted the reducing substance. The determinations made on the samples from these animals are given in Table 10.

TABLE 10

Tests performed on the six beagles of this experiment

	Dog number					
	147	151	302	303	422	424
Protein (Albustix)	X	X	X	X	X	X
Protein (3% acetic acid and heat)	X	X	X	X		
Protein (sulfosalicylic)	X	X	X	X	X	X
Reducing substances	X	X	X	X	X	X
Glucose (Clinistix)	X	X	X	X	X	X
Ketone bodies (Ketostix)	X	X	X	X	X	X
Glucuronic acid	X	X	X	X		
Urea N, urinary					X	X
Urea N, blood					X	X
Creatinine, urinary					X	X
Creatinine, blood					X	X

All dogs developed a slight proteinuria, i.e., they excreted approximately 30-100 mg% daily. Occasionally one of the animals excreted 300 mg%, but this was the maximum amount excreted by any surviving animals; terminally, the urine of dog 303 had a protein concentration over 0.6%. Mild ketosis also occurred occasionally. There were no major changes in the excretion of glucuronic acid. Neither urinary creatinine nor urea N showed any great deviation from control values. However, blood urea nitrogen (BUN) and blood creatinine values did show some changes, as indicated in Figures 16 and 17.

Figure 16. Blood urea nitrogen values in dogs number 422 and 424 after 325 r of Co^{60} γ -irradiation. The average control values were 11.2 mg% (range 10.85 - 11.40 mg%) for dog 422, and 11.8 mg% (range 9.9 - 14.0 mg%) for dog 424.

Figure 17. Blood creatinine values in dogs number 422 and 424 after 325 r of Co^{60} γ -irradiation. The control values for the two dogs averaged 12.5 mg% (range 0.51 - 1.72 mg%).

Semisynthetic Dog Diet*

A semisynthetic dog diet that has been fed to several dogs for the past $2\frac{1}{2}$ years has been revised. Our present formulation (called CASD) is given in Table 11.

TABLE 11

Composition of semisynthetic diet (CASD)

Component	%
Sugar	43.3
Salt mix*	10.0
Casein	30.0
L-Arginine hydrochloride	1.0
L-Methionine	0.2
L-Cystine	0.5
Cellulose	4.0
Vitamin mix (CASB)**	1.0
Cottonseed oil	10.0
Vitamin E Beadlets †	16 g per 10 kg diet

*The trace element mixture incorporated into our salt mix has been increased from 40 to 70 g/10 kg of salt mix.

**Our formulation.

†Hoffman-LaRoche and Co., Nutley,
New Jersey.

Arginine, methionine and cystine were added to supplement the amino acids of casein and to bring the ratios of the essential amino acids more closely in line with those of egg albumin. (Egg protein is the usual reference standard since it is utilized as completely as any other food protein by mammals, and supplementation with additional amino acids does not significantly increase its biological value.) The trace elements were increased following comparison with some commercially available dog foods and experimental diets. The primary result of this change is an increased iron intake.

During this period 7 dams came into heat (3 of them for the first time), and attempts were made to breed all of them. In 5 of the attempts mating did not occur, probably because of the relatively young age of the dogs and their inexperience rather than because of any dietary influence. Of the two successful attempts, the first dam, who had been transferred to the semisynthetic diet 2 years ago from the stock colony, was bred to a

---

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

male who had been weaned to the diet $2\frac{1}{2}$ years ago. This cross produced 5 puppies. Two survive to this date; the other 3 died within a few days of birth. At necropsy, the dead pups were grossly normal. This dam has produced three litters on the diet; the first litter was by normal birth, the second required cesarean section, and the third was normal. The second dam, an offspring of a dog that received the semisynthetic diet, produced a litter of at least 3 pups, but unfortunately cannibalized the litter. The remains of 2 pups showed that they had been stillborn. Attempts at breeding animals born and reared on the semisynthetic diet will continue.

At the time of reporting, 21 dogs are being maintained upon this diet.

Parasitism in the Dog Colony*

Gastrointestinal parasites constitute a problem when the animals are to be used for acute radiation studies. Only the Ascaridae (round worms) are a matter for concern, because our laboratory housing is not particularly favorable for the propagation and transfer of the other families. At present all of the infested animals except two harbor ascarids only; the two exceptions are infested with Ancylostoma (hookworm). This report deals only with the problem of ascarid infections.

Ascarid ova were found in 41% of the fecal specimens from 226 dogs in October and November 1961. In March and April 1962, 28.2% of 255 samples were positive. In June of 1962, dogs that had been negative for three successive previous examinations were excluded; thus only 184 samples were examined of which 32% were positive (or 23.7% of all the dogs). An examination of fecal samples from 293 dogs in September 1962 was made and 21.5% of these samples contained ascarid ova. A limited survey made in December 1962 through January 8, 1963 included 160 dogs that were not negative in all of 3 prior examinations and 40.6% of these 160 (or 22.9% of 283 weaned and adult dogs) were positive.

With only one exception, all the dogs which are presently positive for ascarid infection were born since April 1961. The infected dogs are distributed rather evenly throughout all four wings which constitute the kennel area. However, it appears that most of them become infected in the prenatal or the postnatal period while in the P wing breeding kennels since new infections generally occur in immature or young dogs.

Records of the 61 dogs with evidence of ascarid infection in December 1962 have been examined. In 18 cases it appears that the dogs could have been infected by their dams, since the latter were infected prior to or during the times the dogs were being suckled. In the other 43 cases, the

---

*This work was performed with the assistance of W. G. Richard.

dams were free of evidence of parasitism for at least 10 months and 2 examinations prior to the birth of the pups. Seventeen of these 43 dogs were born of dams that have never had evidence of parasitism.

There is considerable evidence that puppies become infected with ascarids in utero, even when fecal specimens from the dam do not contain ascarid ova. Probably the dams at this time are being infected or reinfected and the fetal infection occurs from migrating larvae. However, only 29.5% of the present cases appear likely to have been infected in utero or by direct contact with the dam; 70.5% probably were infected through environmental contacts after weaning.

An Automated Data Read-out and Computation System for Gamma Spectroscopy*

The large volume of data obtained from multichannel pulse-height analyzers necessitates the use of automatic data-processing equipment. The system described here was developed to meet the current need for rapid analysis of routine measurements and is capable of further sophistication to handle more complicated problems of analysis. It has demonstrated the high degree of reliability that can be obtained with the punch-card system and the rapidity and accuracy with which resultant answers can be obtained. The approach was governed completely by the ready access to a card-fed IBM 1620 computer.

A transistorized 400-channel pulse-height analyzer (RIDL, Radiation Instrument Development Laboratory) is used as the basic gamma spectrometer. The output of this analyzer is routed through a parallel to serial converter (RIDL) that drives an IBM 526 keypunch. This punches standard IBM punch-cards that can be used directly as the input to the computer. The system presently provides an input to the keypunch from 3 remote analyzer locations. An automatic switching mechanism constructed by the ANL Electronics Division permits fully automatic recycling of each analyzer and provides necessary crosstalk between the keypunch master unit and the remote analyzer locations.

For each card the analyzer supplies 10 channels of data and the address of the first channel. The remote-control station supplies an identification denoting the analyzer furnishing the data and a sequential numbering system which advances with each read-out cycle. The keypunch autoduplicates the data on each card. A lead card, manually keypunched for each instrumental reading, contains sample identification and computing factors. The lead cards are collated with the data cards, each run being uniquely identified by the date and serial run number.

---

*This work was performed with the assistance of N. D. Kretz.

A program to accommodate these cards has been written for the IBM 1620 by Mr. M. Dipert of the Computing Section of this division. To date the principal operation has been the analysis of large amounts of data from single radionuclides in dogs and from tissues obtained at necropsy. The computer sums a specified number of channels in a photopeak which may float within a broader given area of interest. Both the number of channels summed and the area of interest may be readily changed by one card in the program deck. The floating summation was necessary to accommodate instrumental drift and changes in the shape of the peak due to absorption and scattering by the sample. Upon reduction of all sums to a per-minute time basis, the average of all background sums in the series is subtracted from the average standard sums for the series and the individual sample sums. From a library of values of identified standards for a specific date, the quantity of the appropriate standard for the date of measurement is computed according to physical decay of the isotope. By comparison of the standard with each sample the quantity of each sample is computed. This is corrected by appropriate absorption and geometric factors to result in the absolute quantity of the particular radionuclide in each sample. The program provides for correction, on a weight basis, of the amount in the measured sample to the amount in the total tissue when the measured sample is less than the total tissue. From this computation the specific activity is obtained. The data and the quantity administered is obtained from a library of radionuclide administration cards, and the time since administration and the fraction of administered quantity retained is computed.

The above-described system of data handling has permitted the rapid analysis of a large amount of data while requiring a fraction of the manpower that would be required using non-machine computations. The present system is being expanded to utilize computer methods of spectrum-stripping and analysis in conjunction with the above data handling.

References

1. Strominger, D., J. M. Hollander, and G. T. Seaborg. Table of isotopes. Rev. Mod. Phys. 30, 585-904 (1958).
2. Anderson, E. C., and B. E. Clinton. Variation of human potassium concentration with age. Biological and Medical Research Group Semiannual Report, Los Alamos Scientific Laboratory. LAMS-2526 (1960), pp. 121-128.
3. Richmond, C. R., and J. E. Furchner. Potassium content of dogs, calculated from natural potassium-40 levels. Biological and Medical Research Group Semiannual Report, Los Alamos Scientific Laboratory. LAMS-2426 (1960), pp. 41-49.

PROGRESS REPORT: TUMOR DEVELOPMENT FOLLOWING INJECTION OF CARCINOGENS AT MULTIPLE SITES

Austin M. Brues, Harry Auerbach and Georgia DeRoche

It may be asked whether the probability of carcinogenic action of a radionuclide deposited in the tissues of an individual - a probability the degree or existence of which implicitly underlies any assignment of permissible levels of exposure - should be assumed to depend on the absolute amount of deposited nuclide, or on the amount relative to body weight.

This question is an academic one as long as permissible levels are based on the calculated radiation dose or dose rate to some "critical" organ or volume of tissue; and it is irrelevant when the permissible dose for man is derived from experience with the morbid results of deposition in man. However, when the permissible level is based to some extent on experimental observations in other species (ordinarily the mouse) the question is a pertinent one.

In comparing different species with respect to the toxicity of bone-seeking radionuclides, the amount administered or retained is usually expressed in relation to body or skeletal mass ($\mu\text{c}/\text{kg}$). On this basis the carcinogenic effect, in terms of mean number of bone tumors produced in a given number of animals, is roughly equivalent in most species regardless of their size.

Since the number of cells at risk differs greatly in different species and is about proportional to the size of the animal, one might expect that a much larger species would be much more susceptible to tumor formation as a result of an equivalent dose relative to body mass, but this is clearly not the case. Since it is not, it seems to follow that a given dose of an insoluble radionuclide, deposited locally, should be much less likely to give rise to a tumor in a large animal than in a small one. This would be consistent with the well-established fact that malignant tumors are more readily produced locally in mice by relatively small doses of carcinogenic hydrocarbons than they are by much larger doses in large animals. Since local tumors may be induced in mice by single injections of insoluble radioactive agents in the same dosage range as the human total-body permissible level, this question has practical as well as theoretical interest when one is called upon to determine how radical a treatment is required for wound contamination, as well as in the more general matters of the hazard of "hot spots" and the use of the "critical organ" concept in the derivation of permissible exposure levels.

This might be tested by giving a wide range of single-injection doses of a locally retained carcinogen to various species. Since, however,

the degree of exposure of individual cells to the carcinogens would not be comparable or indeed susceptible of very accurate estimation, a series of experiments was set up in which different species received identical injections, the number of which was in approximate proportion to the weight of the animal: thus, except for some differences that might be anticipated in the final distribution of the injected carcinogen in the different species, the important difference in exposure would be in the number of cells exposed at a given level of chemical or radioactive insult. In these experiments, to be reported later, mice received a single injection, while rats received 8 such injections in different sites, and rabbits 80 such injections, given at one time. In these experiments, 20-methylcholanthrene (MCA) and Pu^{239} were given subcutaneously.

In experiments employing multiple injections, it is important to establish whether one tumor or one site of injection has any significant influence on the development of a tumor at another site. The present report deals with the results of administering 1, 2, or 8 injections of 0.25 mg of MCA subcutaneously into Sprague-Dawley and Wistar rats.

Materials and Methods

All rats were injected with a 0.5% solution of MCA in trioctanoin (Tricaprylin). Each injection contained 0.25 mg MCA in 0.05 ml of the solvent; controls received 0.05 ml of the solvent. Rats receiving 1 injection were injected subcutaneously in the right flank about 2 cm from the midline; those with 2 injections also received one in the left flank; 8 injections were given, 4 in each flank, 2-2.5 cm apart.

One hundred and thirty-five Sprague-Dawley (SD) female rats and 28 pathogen-free female Wistar (WPF) rats were injected according to the schedule given in Table 12.

TABLE 12

Schedule of injection of rats

Strain	Number of injections	Age, days	Number of rats injected	
			Experimental	Control
SD	1	72	42	15
SD	2	72	28	14
SD	8	56	24	12
WPF	8	125	20	8

All rats were kept 6 in a cage and maintained on commercial rat chow and water ad libitum. They were weighed and examined for palpable tumors at weekly intervals, beginning at the time of injection. Masses were first recorded when they reached a maximum diameter of 5 mm, and biweekly measurements were made on many of them. Three dimensions were measured with calipers, and tumor volumes were calculated as if each were a sphere having a diameter the mean of the three measurements. All animals were kept until death, at which time tumors and grossly abnormal tissues were removed and prepared for histologic examination. All tumors were examined microscopically for classification of tumor type.

Not all the tumors were measured frequently enough to construct growth curves. Where a sufficient number of measurements was recorded, growth curves were made by plotting the logarithm of the tumor "volume" against time. This function approximated a straight line, and the growth rates were expressed by the slopes of these lines, as log volume per day. (Thus, a slope of 0.03 represents a 10-day doubling time.)

Results

It is well known that most female laboratory rats are very susceptible to benign mammary tumors (fibroma, adenoma and more frequently mixtures of the two elements) after the first year of life. Although total-body radiation,⁽¹⁾ external irradiation,⁽²⁾ and ingestion of large doses of MCA by young rats⁽³⁾ have induced mammary carcinomas, such tumors have not appeared in the present study to result from single or multiple local injections of MCA.

About 45% of all control animals showed mammary tumors before death. The incidence of mammary tumors in the experimental animals not developing sarcoma was about 60%. These occur late in life. From Table 13 it can be seen that the average time to first appearance of benign mammary tumor among controls is over 500 days and among those experimental animals developing benign mammary tumors the average is also over 500 days. These benign tumors were typically slow-growing to death of the animal whether in the control or experimental animals.

Methylcholanthrene, in the dose used, was very effective in producing sarcomata in the Sprague-Dawley and Wistar female rats (Table 14). In the 1- and 2-injection animals, 61.9% and 60.7% developed malignant tumors, whereas in the 8-injection Sprague-Dawley and the 8-injection Wistar rats, 91.7% and 85.0% developed malignant tumors. The difference in tumor incidence rates between the combined 1- and 2-injection animals compared with combined Sprague-Dawley and Wistar 8-injection animals is statistically significant ($P = .001$). Failure to detect differences between the 1- and 2-injection groups may be due to the size of the groups used.

TABLE 13

Tumor latency and growth rate in control rats and in rats given 1, 2, or 8 injections of MCA

Strain	Number of injections	Total number of tumors	Mean days to first appearance of tumor	Survival, days after injection	Mean slope of growth
Malignant tumors					
SD	1	25	253.3	320.6	0.03158 (5)**
SD	2	18	259.9	343.6	0.02829 (11)
SD	8	29	166.3	241.4	0.05012 (22)
WPF	8	18	182.4	270.3	0.04010 (14)
Benign mammary tumors					
SD	1	11	516.4	759.7	0.00709 (9)
SD	2	6	528.2	713.0	0.00675 (4)
SD	1C*	9	518.7	791.9	0.00770 (6)
SD	2C	8	441.3	664.1	0.00705 (8)
SD	8C	6	581.0	737.2	-
WPF	8C	3	580.0	845.3	-
Dead with no tumors					
SD	1	5		514.0	
SD	2	6		404.2	
WPF	8	1		665.0	
SD	1C	6		718.0	
SD	2C	9		637.4	
SD	8C	6		684.8	
WPF	8C	5		744.4	

*C denotes controls, injected with solvent only.

**Figure in parentheses gives number of slopes on which average is based.

TABLE 14

Tumor incidence in control rats and in rats given 1, 2, or 8 injections of MCA

Strain	Number of injections	Number of animals	Number of animals with specified tumor(s)								Total number of tumors	Dead with no tumors
			Mal* only (1)	Mam* only (2)	Mal + Mam (3)	Mmal* (4)	Mmam* (5)	Any Mal (1) + (3) (6)	Any Mam (2) + (3) (7)	Any tumor (8)		
SD	1	42	26	11	0	2	4	26	11	37	43	5
	1C*	15	1	7	1	0	4	2	8	9	16	6
SD	2	28	17	5	0	2	2	17	5	22	28	6
	2C	14	0	5	0	0	3	0	5	5	9	9
SD	8	24	17	2	5	11	1	22	7	24	46	0
	8C	12	0	6	0	0	0	0	6	6	6	6
WPF	8	20	17	2	0	9	1	17	2	19	30	1
	8C	8	0	2	1	0	1	1	3	3	5	5
All control		49	1	20	2	0	8	3	22	23	36	26
Per cent values												
SD	1	100	61.9	26.2	-	4.8	9.5	61.9	26.2	88.1	-	11.9
	1C	100	6.7	46.7	6.7	-	26.7	13.3	53.3	60.0	-	40.0
SD	2	100	60.7	17.9	-	7.1	7.1	60.7	17.9	78.6	-	21.4
	2C	100	-	35.7	-	-	21.4	-	35.7	35.7	-	64.3
SD	8	100	70.8	8.3	20.8	45.8	4.2	91.7	29.2	100.0	-	-
	8C	100	-	50.0	-	-	-	-	50.0	50.0	-	50.0
WPF	8	100	85.0	10.0	-	45.0	5.0	85.0	10.0	95.0	-	5.0
	8C	100	-	25.0	12.5	-	12.5	12.5	37.5	37.5	-	62.5

*Mal malignant, Mam mammary, Mmal multiple malignant, Mmam multiple mammary

*Control received injections of solvent only

The average time to first appearance of malignant tumor (0.5 cm mass) in the MCA-treated groups was considerably shorter than that observed for benign mammary tumors (Table 13). Similarly, the average days from first appearance of tumor to the death of the animals was reduced compared to benign mammary tumors. In the 1- and 2-injection groups, the average time to first appearance of malignant tumor was 255 days and the average after-survival about 80 days. The two 8-injection groups showed an even shorter average time to first appearance of about 170 days although the average after-survival was again about 80 days.

Table 13 also gives the results of measurement of tumor growth rates. It is seen that the growth rates of the benign and malignant tumors are quite different; the mean doubling time in volume of the malignant tumors in the various groups ranged from 6 to 10 days and that of the benign tumors in all groups approximated 40 days. It is interesting to note that the 8-injection experimental animals showed a lower average days to first appearance of tumors compared to the 1- and 2-injection groups and tended to show a slightly higher rate of increase of tumor volume as shown by the larger slopes.

The average time to first appearance of malignant tumors and to subsequent death was so much shorter than that for the benign mammary tumors that very little overlap in the two types of tumors could occur (Figure 18). Thus very few of the animals with malignant tumors ever

Figure 18. Time to first appearance of tumor and time to death in control rats and in rats given 1, 2, or 8 injections of MCA.

developed a mammary tumor, although MCA-injected animals not developing malignant tumors developed the normal control incidence of mammary tumors. The incidence of multiple malignant tumors varied from 4.8% in the 1-injection group to 7.1% in the 2-injection groups, 45.8% in the 8-injection SD group and 45.0% in the 8-injection WPF group (Table 14). The difference in multiple malignant tumor incidence rates between the 2-injection group and the combined 8-injection groups is statistically significant ($P = .001$). The occurrence of multiple malignant tumors in the 1-injection group may be attributed to the natural incidence of about 6% malignant tumors as seen in the control populations. Failure to observe a significant increase in multiple malignant tumors in the 2-injection group may be due to the sample size.

It is to be expected that a higher total incidence of malignant tumor and a higher incidence of multiple malignant tumors should have occurred in the multiple injection groups in view of the 60% incidence of malignant tumor in the 1-injection group. In the 8-injection animals only one rat was observed with 4 malignant tumors. All the other animals having multiple malignant tumors showed either 2 or 3 tumors. Because of the rapid growth of the sarcomata and the relatively short period between first appearance of tumor and death, the number of 8-injection animals having multiple tumors observed must be less than the theoretical number. The period of time between first appearance of a sarcoma and the death of the animal was in most cases not of sufficient length to allow for the development or observation of subsequent tumors from the other injection sites.

The distribution of tumor appearance times (days after injection) in the rats given single MCA injections is shown in Figure 19. Most of these

Figure 19. Distribution of times of first appearance of tumors in rats given single injections of MCA.

times follow a log normal distribution except at the two extremes. In order to make a rough estimate as to whether the fate of the animals receiving 2 or 8 injections was comparable to that of randomly chosen groups of 2 and 8 singly injected rats, the actual data in the single-injection experiment were used.

The time to appearance of the tumor and the time to death were recorded on each animal and the order of these animals was rearranged 50 times by the use of a table of random digits. From each of these sorts, 2 groups of 8 and 2 groups of 2 each were

marked off and from each of these groupings the time of appearance of the first tumor and the time to death of the first animal were obtained. The

means of these values for the several groups of 2 and 8 animals appear as "calculated" days in the first two rows of Table 15 alongside the values found in the experiments in which the corresponding number of injections was given to single animals.

TABLE 15

Expectations for two injections and for eight injections, derived by randomly assorting data on the singly injected rats, and calculating from 100 passes

	One injection	Two injections		Eight injections	
		Found	Calculated	Found	Calculated
Mean days to appearance of malignant tumor	253.3	259.9	215.5	166.3*	148.6
Mean days to death with malignant tumor	320.6	343.6	294.8	241.4	208.8
Mean number of malignant tumors per rat	0.58	1.00	1.03	1.92	2.48
Mean number of mammary tumors per rat	0.34	0.20	0.17	0.17	0.05
Rats with multiple malignant tumors, %	4.8	7.1	16.0	45.8	86.0
Rats with no tumors, %	12.0	23.0	4.0	0	0

*Standard deviation 59.3; standard error of the mean 11.0.

Then, considering each of the separate groups as if it were a single animal developing tumors in the order they appeared in the group, terminating with "death" at the time of the first death in the group, the other calculated values in Table 15 were derived. The differences between the observed and calculated times and multiplicity of tumors are such as to suggest an increased latent period and a decreased incidence of multiple malignant tumors after multiple injections relative to expectation from the results of single injections; however, these differences are not striking and may be fully attributable to sampling error. The differences are not such as to seriously impair the interpretation of multiple injections in experiments in local carcinogenesis.

Summary

1. Methylcholanthrene subcutaneously injected into female rats in highly carcinogenic doses (0.25 mg) has no observable effect on the time of development, growth rate, or benign nature of the benign mammary tumors that are common in these animals.
2. The induced malignant tumors (ordinarily sarcomas) are clearly distinguished from the benign mammary tumors by their time of appearance and their growth rate.
3. When multiple injections are given, the latent period to first appearance of a tumor is shortened; the mean number of tumors in a group of rats and the proportion of rats with multiple tumors is increased. This can largely be explained on a statistical basis: i.e. as if each tumor develops as it would in a singly-injected rat, independently of other sites of injection or other developing tumors. There may, however, be a slight retardation of tumor development from this expectancy in animals receiving 8 injections at the same time.

References

1. Shellabarger, C. J., E. P. Cronkite, V. P. Bond, and S. W. Lippincott. The occurrence of mammary tumors in the rat after sublethal whole-body irradiation. *Radiation Res.* 6, 501-512 (1957).
2. Passonneau, J. V., A. M. Brues, and K. F. Hamilton. Superficial carcinogenesis by distributed and point sources of beta radiation. *Proc. Am. Assoc. Cancer Res.* 1, 41 (1953).
3. Huggins, C. and N. C. Yang. Induction and extinction of mammary cancer. *Science* 137, 257-262 (1962).

DYNAMICS OF TUMOR GROWTH

Anna Kane Laird

In recent work^(1,2) we have shown that the growth of many tumors can be described empirically by a straight line on a log-log plot, that is, that the log of tumor size increases directly with the log of time, to a close approximation.

Mathematically a log-log plot is described by a power function of the form $y = ax^b$; in this case y is tumor size, x is the time during which the tumor has grown, and a and b are constants. In its nature a power function can be thought of as a series of exponential functions of alternating sign. However, since the growth of tumors must occur mainly by proliferation of already existing tumor cells, such a function is difficult to relate to the biological processes underlying tumor growth.

In the present paper we will show that the observed growth of most tumors can be schematized as an exponential proliferation of tumor cells modified by an exponentially increasing retardation, which is evident throughout the growth of the tumor.

Analysis of Tumor Growth

If the divisions of tumors cells were to occur at constant intervals on the average, we would observe simple exponential growth of the entire tumor mass. Even if a constant proportion of the new tumor cells died in each generation, the growth curve would be exponential, although the rate of such exponential growth would be slower (the slope of the semilog line would be reduced). However, the empirical finding that for tumor growth to fit a straight line, the x -axis (time axis) as well as the y -axis (axis of tumor size) must be contracted on a logarithmic scale, suggests that in these cases a retardation of tumor growth occurs that is itself exponential. Indeed, when one measures on any plot of tumor growth the times required for successive doublings of tumor size, one finds that these doubling times increase quite regularly, in an exponential fashion. Growth of this form is described mathematically not by a power function but by a Gompertzian function:

$$W = W_0 e^{A/\alpha(1 - e^{-\alpha t})}$$

where W is tumor size at time t , W_0 is the initial tumor size, and A and α are constants. In this function the exponential cell proliferation that we assume to be occurring is represented by the lower exponential function,

while the exponentially increasing delay in the doubling time is represented by the upper exponential function, itself the exponent of the first term. The constant A measures the initial reproductive rate, and the constant α measures the continuing retardation in this rate as the tumor grows. The ratio A/α is a measure of the asymptotic limit of the growth process.

This Gompertzian function has been fitted to the growth measurements of a number of tumors; examples are shown in Figures 20 and 21, which also include arithmetic plots of the semilog, cube root or log-log curves suggested by earlier authors for the same data. It is clear from these graphs that a Gompertzian function follows significantly longer periods of growth for these tumors than does a simple exponential function (semilog curve, Figure 20), a cube root curve (Figure 20), or a power function (log-log line, Figure 21). It is also clear from inspection of the original data points that tumor growth tends to slow continuously, approaching an asymptotic limit. None of the various mathematical functions previously suggested for tumor growth approaches an asymptote, and hence cannot follow the deceleration that is usually observed during tumor growth; the Gompertzian function does follow this deceleration, because it is an asymptotic function.

Figure 20. Growth of the Krebs ascites carcinoma. Arithmetic plot showing a semilog curve fitted to the first 3 points, a cube root curve fitted to the first 17 points, and a Gompertzian curve fitted to all 19 points. Data of Patt and Blackford.⁽³⁾

Figure 21. Growth of the DBA ascitic lymphoma. Arithmetic plot showing a semilog curve fitted to the first 3 points, a log-log curve fitted to the first 7 points, and a Gompertzian curve fitted to all 11 points. Data of Revesz and Klein.⁽⁴⁾

In Table 16 are given the values computed for the Gompertzian constants A , α and W_0 , for a number of the tumors reported in the literature. In all cases an arbitrary small value was first assigned to W_0 to permit computation of an approximate value for A and α . It was then possible to compute a value for W_0 by assigning these values of A and α as starting values and computing the three parameters simultaneously. The data in most cases were weighted by the reciprocal of the tumor size for each point.

TABLE 16

Gompertzian analysis of tumor growth values for constants A , α , and W_0

Tumor	Reference	A	α	W_0
Krebs	(3)	5.25 ± 2.00	0.411 ± 0.056	2710 ± 8788 cells
Ehrlich	(5)	0.078 ± 0.011	0.0090 ± 0.0008	$426,376 \pm 274,956$ cells
MC ₁ M, low dose	(5)	0.119 ± 0.004	0.0147 ± 0.0006	$139,134 \pm 697$ cells
6C ₃ HED, high dose	(4)	0.0397 ± 0.003	0.012 ± 0.0015	$50 \times 10^6 \pm 1.97$ cells
6C ₃ HED, low dose	(4)	0.0626 ± 0.0062	0.0116 ± 0.0021	$10 \times 10^6 \pm 12.87$ cells
DBA lymphoma	(4)	0.276 ± 0.023	0.0238 ± 0.0021	$10,000 \pm 5890$ cells
El ₄ , low dose	(4)	0.207 ± 0.096	0.019 ± 0.003	$23,916 \pm 8280$ cells
El ₄ , high dose	(4)	0.172 ± 0.097	0.023 ± 0.004	$695,424 \pm 1.99 \times 10^6$ cells
Osteosarcomas	(6)	1.02 ± 0.115	0.159 ± 0.026	0.01 ± 157 cm ³
Walker 256 (W26b1)	(7)	0.220 ± 0.0227	0.0218 ± 0.0061	0.4 ± 130 grams
Walker 256 (W12a7)	(8)	0.342 ± 0.040	0.0205 ± 0.0058	1 ± 15.274 mm ³
Walker 256 (W10b4)	(8)	0.132 ± 0.012	0.003 ± 0.0026	4 ± 2646 mm ³
Walker 256 (W10a6)	(8)	0.362 ± 0.017	0.039 ± 0.0037	100 ± 154 mm ³

The values for A lie generally between about 0.08 and 0.36, and for α between about 0.01 and 0.02, but several exceptions stand out, notably the high values for A and α found for the Krebs tumor, and the very low value for α found for one of the Walker 256 tumors. The ratio A/α , which measures the asymptote of the growth curve, is remarkably constant in spite of the differences in the individual values for A and α .

Although the values computed for W_0 in many cases seem large, they are extremely small in terms of the scale of the growth process as a whole; except for the 6C₃HED, high dose, the values computed for W_0 are not greater than 0.1% of the cell numbers finally approached in the growth of these tumors. That is, these curves cover in nearly all cases more than a 1000-fold increase in size of the tumor.

The Gompertzian function and the model of tumor growth described by it can both be shown to include simple exponential growth as a special case: In the model, if the ratio of successive intervals between doublings of the tumor mass approaches 1.00, then the doubling times become constant, as is characteristic of simple exponential growth.

For the Gompertzian function to describe simple exponential growth, in which successive doubling times remain constant, it is evident that α ,

the factor by which successive doubling times increase in our model, must be reduced to zero. This relation between the Gompertzian and a simple exponential function can be demonstrated in the following way:

Starting with the Gompertzian function $W = W_0 e^{A/\alpha (1 - e^{-\alpha t})}$, we expand the term $e^{-\alpha t}$ to a power function:

$$e^{-\alpha t} = 1 - \alpha t + \frac{\alpha^2 t^2}{2!} - \frac{\alpha^3 t^3}{3!} + \dots$$

Substituting in the exponent of the Gompertzian function we have

$$A/\alpha \left(1 - 1 + \alpha t - \frac{\alpha^2 t^2}{2!} + \frac{\alpha^3 t^3}{3!} - \dots \right)$$

or

$$A/\alpha \left(\alpha t - \frac{\alpha^2 t^2}{2!} + \frac{\alpha^3 t^3}{3!} - \dots \right)$$

Dividing through by α we obtain

$$A \left(t - \frac{\alpha t^2}{2!} + \frac{\alpha^2 t^3}{3!} - \dots \right)$$

and thus $W = W_0 e^{A \left(t - \frac{\alpha t^2}{2!} + \frac{\alpha^2 t^3}{3!} - \dots \right)}$

Then if $\alpha = 0$, the Gompertzian function becomes $W = W_0 e^{At}$, which is a simple exponential equation.

Discussion

Division of one cell to produce two cells, and of the two cells thus produced to form four cells, and so on, at some constant mean interval, constitutes the usual situation in many cultures of free-living bacterial or tissue cells while conditions of nutrition are optimum. It also represents the expected theoretical finding implicit in the mitotic division of cells. In such a system, if a constant proportion of cells died in each generation, the rate of net growth of the population would be decreased, but the dynamics of growth would not be altered.

It is evident that the observed increase in the time required to produce a doubling of the mass of a tumor could result equally well from a regular increase in the proportion of cells lost from the reproductive population in each generation, or from a regular increase in the mean generation time with successive generations. In either case, to produce the observed effect on the growth of the tumor as a whole, the magnitude of the retarding factor must increase exponentially with time, since the deceleration in tumor growth is exponential. An exponentially increasing proportion of dying cells should be observable; however, Klein and Revesz,⁽⁵⁾ studying the Ehrlich ascites tumor, and Patt and Blackford,⁽³⁾ studying the Krebs ascites carcinoma, both noted that the proportion of non-viable cells in these tumors is very low and does not change significantly with time. If this finding proves to be true of other tumors, we are left with two other hypotheses. 1) that the mean generation times tend to increase during the growth of many tumors for reasons at present unknown or 2) that cells may be lost from the exponentially reproducing population but remain viable.

It must be stressed that the retardation implied by the Gompertzian function is present from the earliest observations of tumor growth, and increases regularly throughout the life of the tumor. This retardation must be distinguished from the relatively sudden and more extreme retardation observed very late in the growth of many tumors; in some cases the tumor may even get smaller after reaching a maximum size. This terminal retardation is not accounted for by the Gompertzian function, and we assume that it represents necrosis of the tumor as it outgrows the nutritional support provided by the host. Because the Gompertzian retardation is present throughout growth of the tumor, and because it is mathematically regular, such retardation appears to be an essential quality of the growth of most tumors within the host organism, as opposed to the more or less capricious dissolution of the growth process as the host approaches death.

Use of the Gompertzian function to describe normal growth of the whole organism was first suggested by Wright.⁽⁹⁾ This function shares with several others a number of mathematical properties that enable it to describe the characteristics observed directly in the growth of the whole organism:⁽¹⁰⁾ such functions have limiting values (size does not go on increasing indefinitely); the specific acceleration of growth is always negative (the rate of growth is always decreasing with time), and the specific growth rate decreases more and more slowly as the organism gets older (growth processes tend to approach a final steady state). These are the observable properties of the growth of whole organisms to be sure, but it is surprising to find it possible to describe tumor growth by the same type of mathematical function. It is noteworthy that tumors growing outside the animal host, i.e., in tissue culture, appear to show no such retardation of growth as described by the Gompertzian function;^(11,12) their growth in tissue culture is reported to follow a simple exponential curve closely. Such a finding suggests that

tumors are not nearly so uncontrolled and so foreign when growing within the host organism as we have been accustomed to think; that perhaps the organism as a whole exerts a controlling effect on the growth of its parts, including its tumors, keeping them within some characteristic limit.

References

1. Laird, A. K. Dynamics of tumor growth. Proc. Am. Assoc. Cancer Res. 3, 336 (1962).
2. Laird, A. K. Dynamics of tumor growth. I. Power function analysis. Unpublished.
3. Patt, H. M. and M. E. Blackford. Quantitative studies of the growth response of the Krebs ascites tumor. Cancer Res. 14, 391-396 (1954).
4. Revesz, L. and G. Klein. Quantitative studies on the multiplication of neoplastic cells in vivo. II. Growth curves of three ascites lymphomas. J. Natl. Cancer Inst. 15, 253-271 (1954).
5. Klein, G. and L. Revesz. Quantitative studies on the multiplication of neoplastic cells in vivo. I. Growth curves of the Ehrlich and MC₁M ascites tumors. J. Natl. Cancer Inst. 14, 229-272 (1953).
6. Finkel, M. P., P. J. Bergstrand and B. O. Biskis. The latent period, incidence, and growth of Sr⁹⁰-induced osteosarcomas in CF₁ and CBA mice. Radiology 77, 269-281 (1961).
7. Schrek, R. A comparison of the growth curves of malignant and normal (embryonic and postembryonic) tissues of the rat. Am. J. Pathol. 12, 525-530 (1936).
8. Schrek, R. A quantitative study of the growth of the Walker rat tumor and the Flexner-Jobling rat carcinoma. Am. J. Cancer 24, 807-822 (1935).
9. Wright, S. J. Am. Statist. Soc. 21, 493 (1926).
10. Medawar, P. B., in Essays on Growth and Form, ed. W. E. LeGros Clark and P. B. Medawar, Clarendon Press, Oxford, 1945.
11. Waymouth, C. A rapid quantitative hematocrit method for measuring the increase in cell population of Strain L (Earle) cells cultivated in serum-free nutrient solutions. J. Natl. Cancer Inst. 17, 305 (1956).
12. Puck, T. T., P. I. Marcus and S. J. Cieciura. Clonal growth of mammalian cells in vitro. Growth characteristics of colonies from single HeLa cells with and without a "feeder" layer. J. Exptl. Med. 103, 273 (1956).

PHYSIOLOGICAL EFFECTS OF GLUCOSE OXIDASE IN MICE

Robert N. Feinstein, Marilyn Coulon and John E. Seaholm

The enzyme glucose oxidase catalyzes the reaction

In an attempt to induce continuous production of H_2O_2 in vivo, glucose oxidase was injected into normal mice. The resulting effects on erythrocytes, hematin pigments, and carbohydrate stores proved sufficiently interesting to warrant fuller investigation.

Material and Methods

All animals were CF#1/Anl female mice, 3-5 months of age. In any single experiment, mice used were of the same age, within a week. Four different commercial preparations of glucose oxidase were used: Sigma Chemical Co. (Type III preparation), Dawe's Laboratories* (Glucose Oxidase, Refined), Nutritional Biochemical Corporation, and Enzyme Development Corporation (Deoxin Special). The Dawe product was used except where otherwise stated.

For blood studies, blood was drawn from the orbital sinus by the technique of Riley,⁽¹⁾ using heparinized capillary tubes which could then be used either a) as a source of whole blood by transfer to micropipettes or b) as hematocrit tubes which, after centrifugation, gave not only the hematocrit value but also served as sources of plasma (measurable by volume) and packed cells (measurable by tube length, convertible to volume).

Total hematin pigment and per cent methemoglobin of whole blood, plasma, or packed blood cells were determined by the method of Hunter,⁽²⁾ blood glucose by the method of Nelson,⁽³⁾ and liver glycogen by the method of Good, Kramer, and Somogyi,⁽⁴⁾ followed by the glucose method of Nelson.⁽³⁾

Glucose oxidase assays were performed by a newly devised and unpublished technique which must at present be considered only semi-quantitative. Briefly, the enzyme preparation is incubated with glucose at 37°C in phosphate buffer, pH 6.0, in the presence of sodium azide ($2.5 \times 10^{-4} \text{ M}$, final concentration) to inhibit catalase activity. (Almost all commercial glucose oxidase preparations were found to be heavily

---

*We wish to thank Dr. George E. Ward of Dawe's Laboratories for generous gifts of this product.

contaminated with catalase except Deoxin Special, which shows only traces of catalatic activity.) Action was halted with H_2SO_4 , and the H_2O_2 formed was measured by the addition of TiCl_4 , the yellow color being measured at 420 $\text{m}\mu$ and compared with a standard calibration curve.

For dry weight determinations, tissues were weighed fresh and again after drying to constant weight at 100-105°C. Loss of Evans Blue from plasma was measured in a group of mice of identical weight. Each mouse was given 0.25 ml of 2% Evans Blue solution by intravenous injection via a tail vein; a single syringe and needle were used for the entire group. After 5 min, and again after 35 min, minimal amounts (about 0.1 ml) of orbital sinus blood were withdrawn and centrifuged. After the hematocrit was read, 25 μl of plasma was withdrawn and added to 0.5 ml H_2O in micro-cuvettes matched in the 6-110 semimicro adapter on the Coleman Model 6B spectrophotometer; readings were taken at 600 $\text{m}\mu$.

Results

Toxicity and glucose oxidase activity. The LD_{50} after intraperitoneal injection was determined for each of the four enzyme preparations, and the glucose oxidase activities were determined as described above. It will be observed (Table 17) that although the four preparations vary over 15-fold in lethality and in glucose oxidase activity, the glucose oxidase activity of an LD_{50} is quite constant. This suggests that the toxicity of the preparations is indeed due to glucose oxidase activity rather than to some contaminant.

TABLE 17

Lethality and glucose oxidase activity of several commercial glucose oxidase preparations

Source	LD_{50} , mg/kg	Glucose oxidase activity	Glucose oxidase per LD_{50}
Sigma	4	2.37	9.5
Dawe	13	0.68	8.8
Nutritional Biochemical	21	0.46	9.7
Enzyme Development	70	0.145	10.2

Glucose oxidase activity is expressed as mmols H_2O_2 produced per mg enzyme preparation under standard conditions described in text.

Survival time. It soon became apparent that certain effects observed after injection of glucose oxidase were qualitatively as well as quantitatively dependent on the dose. Therefore, most of the phenomena described below were investigated using three different doses. The enzyme employed was the Dawe product, and this was administered at 50, 250, and 1250 mg/kg. It will be noted (Table 17) that even the lowest of these doses of the Dawe enzyme is approximately 4 times the LD_{50} . The median survival time for animals receiving an LD_{50} is about 2 days; median survival times for mice receiving 50, 250, and 1250 mg/kg were 4.5 hr, 1.5 hr, and 51 min. respectively (Figure 22).

Figure 22 Survival time after various doses of glucose oxidase. Dawe glucose oxidase was injected intraperitoneally into 8-10 mice for each curve. Mean survival time: 51 min after 1250 mg/kg; 90 min after 250 mg/kg; 4.5 hr after 50 mg/kg.

Hematocrit. Effects of glucose oxidase on hematocrit (Figure 23) varied, in extent and even in direction, with the dose. After 50 mg/kg, the hematocrit rose strikingly. Individual values as high as 80 were observed; such blood flows extremely sluggishly, and it is difficult to obtain an adequate sample from the orbital sinus or even by decapitation. After enzyme doses of 250 or 1250 mg/kg, the hematocrit decreased; individual values as low as 7 were seen.

Hematin pigments. Optical absorption at 525 $m\mu$ was used as a measure of total hematin pigments, since according to Hunter⁽²⁾ this wavelength is isobestic* for hemoglobin and methemoglobin. Measurements were made on whole blood, plasma, and packed red cells (after lysis).

Figure 24 presents the effects of the enzyme on the total hematin pigment in whole blood. After a glucose oxidase dose of 50 mg/kg, the total pigment increased in a manner entirely comparable to the increase in hematocrit. After 250 or 1250 mg/kg, pigment values decrease, but to a lesser degree, especially after 1250 mg/kg, than expected from the decreased hematocrit. This discrepancy is due to the fact that, as noted below, the plasma is so highly colored after these doses of enzyme as to contribute heavily to the pigment of the whole blood.

*According to Hunter,⁽²⁾ "an isobestic wavelength is one at which solutions of different pigments at equal concentrations, have identical transmission values."

Figure 23. Hematocrit values after intraperitoneal injection of glucose oxidase. Each point represents average value for 3 mice.

Figure 24. Total hematin pigment of whole blood after intraperitoneal injection of glucose oxidase. Each point represents average value for 3 mice.

Figure 25. Total hematin pigment in blood plasma after intraperitoneal injection of glucose oxidase. Each point represents average value for 3 mice.

Although only traces appeared after doses 50 mg/kg, considerable amounts were observed after the higher doses. In fact, in some cases packed cell level for hematocrit determinations could be measured only by gently lowering into the centrifuged capillary tube a light, footed glass rod and noting its point of initial settling. This heavy pigmentation of plasma is associated with pigment in excreted urine. The internal organs of animals killed at this time were observed macroscopically and microscopically to be packed with pigment. This appeared especially true of the spleen.

Total hematin pigment was also measured in packed blood cells after lysis (Figure 26). The only significant change was at the 1250 mg/kg dose level, where there was a decrease to approximately one-third of the original value in 45 min.

Figure 26

Total hematin pigment in packed red blood cells after intraperitoneal injection of glucose oxidase. Each point represents average value for 3 mice. Cells were measured by length of column in hematocrit tube, then converted to volume by measuring volume of a given length of the same piece of capillary tubing. Cells were lysed in excess distilled water before measuring hematin pigment.

Methemoglobin formation. After doses of 250 or 1250 mg of glucose oxidase per kg, the animals were characteristically cyanotic. Analysis indicated that this was due to the formation of significant amounts of methemoglobin. Values reached 25% of the total pigment in whole blood (Figure 27), and approached 20% in plasma (Figure 28). In no case was methemoglobin detected in packed red cells after lysis.

Figure 27. Methemoglobin formation in whole blood after injection of glucose oxidase. Each point represents average value for 3 mice.

Figure 28. Methemoglobin appearance in blood plasma after intraperitoneal injection of glucose oxidase. Each point represents average value for 3 mice.

Plasma volume and tissue water. The increases in hematocrit and in hematin pigments of whole blood after 50 mg of glucose oxidase per kg suggests a decrease in blood plasma volume, with the lost fluids presumably entering the tissues. This possibility has been examined in two ways. In the first experiment (Table 18), Evans Blue was injected intravenously into a series of normal mice and mice injected with glucose oxidase. The first column of Table 18 shows clearly that the dye is distributed in a lesser volume of plasma in the enzyme-injected mice than in the normal animals.

TABLE 18

Concentration of Evans Blue in plasma

	Initial	Final	Loss
No enzyme	0.514	0.317	0.197
5-35 min after enzyme	0.614	0.360	0.254
3-3.5 hr after enzyme	0.93	0.59	0.34

Glucose oxidase (Dawe). 50 mg/kg intraperitoneally; Evans Blue 2% solution, injected intravenously at 0.25 ml per mouse, from the same syringe and needle, into mice of identical weight. Three mice were used for each test.

Evans Blue concentrations shown are in plasma after centrifugation and are expressed as optical density at 600 m μ after 20-fold dilution with water.

The second set of experiments bearing on the question of a possible fluid shift is summarized in Table 19, which indicates that the dry weights kidney and liver, at least, were a significantly lower per cent of the fresh weight, i.e., had a higher moisture content, after injection of the enzyme.

TABLE 19

Effect of glucose oxidase on water content of tissues

Tissue	Dry weight, % of fresh		Student's "t"
	Control	Experimental	
Liver	39.1	33.0	4.22*
Intestine	21.8	20.5	1.53
Skin	26.4	28.8	0.91
Muscle	25.9	24.9	1.48
Spleen	23.3	22.0	2.36
Kidney	24.7	22.7	4.48*

Each datum represents the mean of 6 animals. Mice were fasted overnight before use. Tissues were weighed fresh and after achieving constant weight at 100-105°C. "Experimental" animals were killed 3 hr after intraperitoneal administration of 50 mg of glucose oxidase (Dawe) per kg body weight. Asterisk indicates statistical significance of $p \leq 0.01$.

Carbohydrate stores. If the injected glucose oxidase remained enzymatically active in vivo, one would expect decreased blood glucose and liver glycogen. Table 20 indicates that such effects are found after any of several doses of the enzyme.

TABLE 20

Effect of glucose oxidase injections on
blood sugar and liver glycogen

Enzyme, mg/kg	Time, hr	Blood sugar, mg/100 ml	Liver glycogen, % of total liver weight
25	0*		3.1
	1.5		2.3
	3		1.3
50	0*	120	3.3
	3	78	0.9
	3*	116	3.0
50	0*	102	4.6
	1	98	3.2
	2	84	2.7
	3	62	1.4
	3*	104	3.7
50	0*		3.9
	1.5		2.2
	3		1.0
1250	0*	120	2.3
	30	30	1.7

*Since experimental animals tended to be anorexic after injection of glucose oxidase, all animals were starved during the experimental period, and some animals (3 hr, no enzyme) were maintained without food as "starvation controls."

Discussion

The question of fundamental interest from our point of view is whether or not the symptoms described are actually attributable to the production of hydrogen peroxide. It seems to us that on the basis of the equation

the syndrome might result from one or more of the following factors:

a) carbohydrate depletion, b) hypoxia, c) gluconate toxicity, d) acidosis from gluconic acid formation, e) H_2O_2 toxicity, or f) toxicity unrelated to the glucose oxidase itself, i.e., toxicity due to a contaminant. While no single experiment has yet been devised to test these possibilities, the evidence at hand suggests that several of them can be eliminated from consideration. Thus, for example, the direct proportionality between the enzyme activity and the toxicity of several commercial preparations of glucose oxidase (Table 17) suggests that the lethality is not due to a contaminant. This, of course, does not demonstrate that some symptomatology may not be due to a contaminant; however, we have observed the same symptoms at comparable dose levels with other brands of the enzyme. Carbohydrate depletion also seems an unlikely explanation since we found that injection of glucose into mice lethally injected with glucose oxidase actually exacerbated, not relieved, the symptoms: hematocrit and pigment changes appeared sooner and to a greater degree, survival time was shortened after lethal doses of the enzyme, and the lethality of approximately midlethal doses was increased.

We have done no experiments bearing directly on the role of hypoxia. However, the syndrome seems totally unrelated to the picture of hypoxic death. We have not yet tested the possible role of acidosis, although we have determined that the maximum amount of gluconate which could theoretically be formed, if administered in the form of the sodium salt, is without toxicity.

These considerations thus favor, by elimination, the concept that H_2O_2 is the active agent. Experiments with catalase inhibitors also provide suggestive evidence bolstering this viewpoint. Sodium azide inhibits not only catalase but also a wide variety of other heme enzymes; 3-amino-1,2,4-triazole (AT) is a fairly specific inhibitor of catalase. Either of these agents, if injected in sublethal doses before glucose oxidase, sharply decreases the LD_{50} of the injected glucose oxidase and shortens the survival time.

If, however, H_2O_2 is the causative agent, the question must then be raised as to how methemoglobin can be formed in the presence of abundant catalase. (We have assayed the catalatic activity of whole blood from animals moribund from glucose oxidase injection; it remains within normal limits.) Three comments may be relevant: First, a comparison of Figures 27 and 28 indicates that methemoglobin never appears in whole blood samples before it appears in the plasma; this, together with our failure to detect methemoglobin in packed erythrocytes, suggests that H_2O_2 may oxidize hemoglobin to methemoglobin only after the hemoglobin has been released from the cells and thus separated from the catalase. Second, a solution of hemoglobin obtained by laking human red cells is completely converted to methemoglobin in about 5 days when exposed to an atmosphere of 95% air and 5% CO_2 .⁽⁵⁾ Third, Hochstein⁽⁶⁾ has recently shown that

methemoglobin may be formed in catalase-rich erythrocytes in vitro in the presence of diffusing H_2O_2 (i.e., low concentrations, continually provided) and in the absence of glucose. Which of these explanations is most nearly relevant has not been decided.

The Evans Blue experiment warrants an extra comment. The only unequivocal conclusion to be drawn from Table 18 is that, at the time of increased hematocrit and increased total blood pigment, the plasma volume is significantly decreased. Although it could also be concluded, from a superficial glance at the data, that the increased 30-min loss of Evans Blue indicates increased capillary permeability, this may well be in error. The increased loss of dye appears rather due simply to the higher initial level in the plasma. If one administers less dye, so that the Evans Blue color of the plasma of enzyme-treated mice is about the same as that of normal mice, the 30-min loss of the dye appears less in the treated than in the normal mice. The dye clearance appears to depend on the balance of a number of reactions too complicated for further discussion here.

It is of interest to try to account for the fluid which apparently leaves the plasma after low, but lethal, doses of glucose oxidase. If we assume that the only significant tissue gains are in liver and kidney (Table 19), and that our mice contained 3.0 ml of blood, with normal hematocrit of 50 and ultimate hematocrit of 75, and with 1.2 g of liver and 0.3 g of kidney,⁽⁷⁾ it can be calculated that approximately 1 g of fluid has left the circulation, while the kidneys gained 0.05 g fluid, and the liver gained 0.22 g. Thus the measured gain in tissue fluid accounts for only a quarter of the fluid lost from the circulation, and it must be considered that the rest of the fluid has been taken up by less easily measurable tissues e.g., fat pads or peritoneal fluid, or perhaps has been excreted.

The finding of a sharp decrease in the total pigment per unit volume of packed blood cells after 1250 mg of glucose oxidase per kg is of interest. No precise mechanism has been established for this phenomenon, but we suggest that it is due to the presence of enlarged but unlysed red blood cells, as well as of some sedimented but pigment-free stromal fragments. Both of these would tend to increase the volume of the packed cell layer without concomitant increase in pigment.

It will be noted that the "normal" values obtained for hematocrit and for total blood pigment are higher than those commonly quoted. This matter was tested, and it was clearly established that orbital sinus blood is significantly higher than decapitation blood in both respects:

	Orbital sinus	Decapitation	Student's "t"
Hematocrit	52.8	49.0	8.09 (d.f. = 11)
Pigment, g/100 ml	15.73	14.99	4.29 (d.f. = 11)

Summary

The physiological effects of glucose oxidase, which oxidizes glucose and produces H_2O_2 , were studied in mice after intraperitoneal injection of supralethal doses of the enzyme.

Four different commercial brands of glucose oxidase were used. Despite a 15-fold variation in activity, the enzyme activity was inversely proportional to the LD_{50} , suggesting that the toxicity is due to glucose oxidase activity, and not to a contaminant.

Intraperitoneal injection of glucose oxidase in supralethal doses causes the appearance of hematin pigment in the plasma, and the appearance of methemoglobin. After high lethal doses of glucose oxidase, the hematocrit decreases, and the total hematin pigment of whole blood also decreases strikingly. After lower, but still lethal, doses of the enzyme, the hematocrit increases, and the percentage of pigment in whole blood correspondingly increases. At least part of this loss of fluid from the blood is accounted for by the finding of increased water content of liver and kidney. After such doses of glucose oxidase, decreases were demonstrated in blood sugar and in liver glycogen.

References

1. Riley, V. Adaptation of orbital bleeding technique to rapid serial blood studies. *Proc. Soc. Exptl. Biol. Med.* 104, 751-754 (1960).
2. Hunter, F. T. The Quantitation of Mixtures of Hemoglobin Derivatives by Photoelectric Spectrophotometry. Charles C. Thomas, Springfield, 1951.
3. Nelson, N. A photometric adaptation of the Somogyi method for the determination of glucose. *J. Biol. Chem.* 153, 375-380 (1944).
4. Good, C. A., H. Kramer, and M. Somogyi. The determination of glycogen. *J. Biol. Chem.* 100, 485-491 (1933).
5. Eder, H. A., C. Finch, and R. W. McKee. Congenital methemoglobinemia. A clinical and biochemical study of a case. *J. Clin. Invest.* 28, 265-272 (1949).
6. Hochstein, P. in discussion: Peroxides in Radiobiology: a Synthesis. *Radiation Research* 18, 296 (1963).
7. Feinstein, R. N., M. Hampton, and G. J. Cotter. Species specificity of catalase. *Enzymologia* 16, 219-225 (1953).

EFFECTS OF HISTOLOGICAL AND HISTOCHEMICAL PROCEDURES ON THE INTENSITY OF THE LABEL IN AUTORADIOGRAMS OF CELLS LABELED WITH TRITIATED COMPOUNDS

Renato Baserga* and Walter E. Kisielewski

Neglecting biological differences in the uptake of tritiated compounds by different types of cells, the label as measured by autoradiography is influenced by a number of factors related to the autoradiographic procedure, such as the method used,^(1,2) the speed of the emulsion,⁽³⁾ the time of exposure and the latent image fading.⁽⁴⁾ However, even with a carefully standardized autoradiographic procedure, the intensity of the label may be modified by variations in the steps preparatory to autoradiography. These include the choice of fixative, and the form in which the tissue is used: sections,⁽⁵⁾ smears⁽⁶⁾ or squashes.⁽⁷⁾ In addition, the intensity of the label may be modified by histochemical procedures selectively removing one or the other of the nucleic acids.⁽⁸⁻¹⁴⁾ The purpose of the present communication is to summarize our findings on the effects of tissue preparation and extractive procedures on the intensity of autoradiographic labeling in terms of mean grain count per cell in cells labeled with tritiated precursors of proteins or nucleic acids.

Materials and Methods

Three 6-month-old Strong A male mice, inoculated 11 days previously with 72 million Ehrlich ascites cells, were injected, respectively, with 60 μ c of thymidine-methyl- H^3 (New England Nuclear Corp., Boston, Mass.) with a specific activity of 3.15 c/mM, 100 μ c of H^3 -cytidine (Schwarz Bio-Research, Inc., Orangeburg, New York), 1.0 c/mM, or 100 μ c of DL-leucine-4,5- H^3 -HCl (New England Nuclear Corp.), 3.57 c/mM. The dose of radioactive isotope was given intraperitoneally in 0.1 ml of sterile water.

The animals were killed after 30 min, and the ascitic fluid was collected in centrifuge tubes and thoroughly mixed with a Vortex Jr. Mixer (Scientific Industries, Inc., Queens Village New York). Aliquots of the tumor cell suspensions were centrifuged, and both the supernatants and the packed cells were analysed according to the procedure described by Feinendegen et al.⁽⁹⁾ Each fraction was first extracted with Carnoy fluid (ethanol, chloroform and glacial acetic acid, 6:3:1) for 30 min, and then

---

*Dr. Baserga (Department of Pathology, Northwestern Medical School) is a Research Development Awardee, United States Public Health Service. This work was in part supported by a grant from the National Institutes of Health.

with 70% ethanol, after which the fraction could be stored; finally, it was extracted with 2% perchloric acid (PCA) for 40 min at 4°C. The supernatants of the 3 extracts were combined to form the acid-soluble fraction,⁽⁹⁾ which represents radioactivity not incorporated into nucleic acids or proteins and removable by the fixatives mentioned below. The residues, containing both nucleic acids and proteins, were redissolved for the determination of radioactivity by liquid scintillation counting, and constituted the amount of radioactivity not removable by fixatives. Air-dried smears from other aliquots of the ascitic fluid were either in methanol or Carnoy; in either case, after fixation, the slides were rinsed in 2 changes of absolute ethanol and one change of 95% ethanol, and again air-dried.

Sections of Ehrlich ascites tumor were prepared by soaking a small fragment of Gelfoam (Upjohn) in a centrifuge tube containing the ascitic fluid. The Gelfoam fragment was fixed as above and embedded in paraffin by routine histological procedures.

In experiments to determine the effects of extractive procedures on the intensity of the autoradiographic label, the following histochemical procedures were performed on smears fixed in methanol or Carnoy fluid. The acid-soluble fraction⁽⁹⁾ was obtained by extraction in a Coplin jar filled with 2% PCA for 40 min at 4°C. Occasionally, this treatment preceded fixation. Free amino acids were removed by 0.5% trichloroacetic acid (TCA) for 1 hr at room temperature.⁽¹⁵⁾ Two methods were used to remove RNA from fixed cells. In the first of these methods, smears were extracted with 10% PCA at 4°C for the desired length of time; in the second method they were digested with RNase (crystallized, salt-free ribonuclease, Worthington, Freehold, New Jersey), 1 mg per 1 ml of distilled water, for 4 hr at 40°C.⁽⁸⁾ DNA was removed from fixed cells with DNase (Worthington), 0.05 mg/ml, in Tris buffer with 0.2 M $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$, adjusted to a final pH of 5.7.⁽⁸⁾ DNase digestion was carried out at 37°C for either 7 or 16 hr. After enzymatic treatment, the slides were extracted in 2% PCA at 4°C for 40 min, and then rinsed in 3 changes of distilled water.

Autoradiograms were prepared according to the method described by Messier and Leblond⁽¹⁾ and by Jofte,⁽¹⁶⁾ using undiluted NTB emulsion (Eastman Kodak Co., Rochester, New York) and exposure times varying from 4 to 21 days. In general, the exposure time was such that the control slides had a mean grain count of 30-50 grains per cell. After processing with D-19 developer and acid fixer (both Eastman Kodak), the slides were stained with Mayer's hematoxylin and eosin.

Mean grain counts per labeled cell were determined on 50 cells of each smear or tissue section, and at least 3 slides were used for each treatment. The limits of accuracy of the method may be obtained by determining the mean grain count per labeled cell in autoradiograms of smears taken from the same tumor sample and treated in the same fashion.

In one of such determinations, on 4 smears from the same tumor pool, the mean grain count, when calculated on 50 cells per smear, varied from 29 to 36, with a mean of 31 for the 4 smears.

With leucine and cytidine, practically all the cells were labeled, and the mean grain count was determined on interphase cells taken at random. With thymidine, the percentage of labeled tumor cells varied from 50 to 60, and the mean grain count was determined by counting only cells that were labeled. For the classification of cells as labeled by tritiated thymidine, the background was determined by counting grains in cells in mitosis, because at the time intervals used in the present experiments these cells show no uptake of thymidine. For example, a smear may have a mean grain count of about 40, with 60 mitoses counted: 25 with 0 grains, 18 with 1 grain, 7 with 2 grains, 4 with 3 grains, 4 with 4 grains, and 2 with 5 grains. In this smear, all cells with 6 or more grains were considered to be labeled. It should further be emphasized that, of one thousand cells, 385 had from 0 to 5 grains, 16 had from 6 to 10 grains, and 598 had 11 or more grains and were therefore labeled beyond a reasonable doubt. Thus, even if cells with 6-10 grains were to be excluded, the percentage of labeled cells would not vary by more than 1.6 per cent.

The radioactivity in the extracts and in the solutions used for fixation or extraction of smears was determined by liquid scintillation counting. In solutions to be so examined, the number of slides fixed or extracted was always such as to maintain a proportion of 1 slide per 10 ml of fluid. While this kind of measurement is not intended to be quantitative, because of variations in the thickness of the different smears, the absence of radioactivity in a solution indicates that the radioactive material is not dissolving in it. Counting was done using a liquid scintillation spectrometer (Tri-Carb Liquid Scintillation Spectrometer Model 314 C, Packard Instrument Company, Inc., P. O. Box 428, LaGrange, Illinois). Refrigeration was set at -9°C , and the instrument was used according to the manufacturer's instructions, after it was established that the counting response was linear over the range encountered in the samples. Discriminators were set at 10 and 50 volts, and samples were counted to a statistical error of less than $\pm 0.5\%$.

Two kinds of liquid scintillant systems were used. In the first, which used dioxane as the solvent, water is held in solution in the ratio of at least 1 ml water per 3 ml scintillant. The system contains 7 g diphenyloxazole (PPO), 50 g naphthalene (NAP), 0.050 g 1,4- d_1 [2-(5-phenyloxazolyl)] benzene (POPOP) and 1000 ml of dioxane. The second, the toluene-ethanol system, holds water in solution in the ratio of 1 ml water per 65 ml of scintillant. It contains 4 g PPO, 0.015 g POPOP, 230 ml absolute ethanol and 770 ml toluene. All solid reagents used were of special scintillator grade obtained commercially. The solvents were of analytic reagent grade. Quenching effects were corrected for, using the internal standard technique, the sample being counted before and after addition of the standard. Tritiated toluene was

used as a standard. Counting efficiencies up to 8% were obtained for solutions containing 20% water with a background of 20-30 cpm.

Results

The distribution of radioactivity in the supernatant, acid-soluble fraction and residue of Ehrlich ascites tumors, 30 min after administration of the radioactive precursor, is shown in Table 21. Only 12% of the total radioactivity due to thymidine was soluble, but with cytidine and leucine, 60% of the total radioactivity was still soluble in reagents commonly used for the fixation of cells and tissues.

TABLE 21

Distribution of radioactivity in aliquots of Ehrlich ascites tumors
30 min after administration of a radioactive compound

Radioactive precursor	Dose, μ c	Radioactivity, μ c/ml of tumor			
		Supernatant	ASF*	Packed cells Residue	Soluble radioactivity,** %
Thymidine	60	0.551	0.339	6.621	12
Cytidine	100	0.759	1.701	1.463	63
Leucine	100	2.628	12.822	5.817	61

*Acid-soluble fraction.

**Radioactivity in the supernatant and the ASF, percentage of total radioactivity.

Effect of fixatives on the amount of radioactivity in cells labeled with tritiated compounds. Smears from each animal were fixed either in methanol or Carnoy; in addition, other smears were treated before fixation with an extracting agent (2% PCA for smears labeled with thymidine or cytidine, and 0.5% TCA for smears labeled with leucine). The results (Table 22) indicate that the intensity of the label in cells incorporating cytidine or leucine was the same for smears fixed in Carnoy or in methanol, with or without prior treatment with PCA or TCA. With thymidine, the intensity of the label was slightly higher in cells fixed with Carnoy, but the difference was not statistically significant. A considerable amount of radioactivity was found to be present in the fixative solutions used to prepare these smears. However, where the smears were treated prior to fixation with PCA (for thymidine and cytidine) or TCA (for leucine), the radioactivity appeared in these fluids, while subsequent passage in methanol

or Carnoy failed to reveal further loss of radioactivity. No radioactivity was found in alcohols or other reagents used following fixation in methanol or Carnoy.

TABLE 22

The effect of fixatives on the intensity of the label in autoradiograms of Ehrlich ascites tumor cells labeled with tritiated compounds

Radioactive precursor	Mean grain count* per labeled cell in smears fixed with			
	Carnoy	PCA or TCA + Carnoy	Methanol	PCA or TCA + methanol
Thymidine	35	37	29	31
Cytidine	18	20	21	21
Leucine	17	16	19	18

*Each value represents the mean of 3 slides.

Measurements of radioactivity were also performed on samples of developer D-19 and the Kodak acid fixer in which Carnoy-fixed smears had been dipped for lengths of time comparable to those used in the processing of autoradiographs. In all instances, the radioactivity of these fluids was not above background.

Carnoy-fixed Ehrlich cells were smaller (mean diameter $13\ \mu$) than methanol-fixed cells (mean diameter $16\ \mu$). The nucleus was more distinct in methanol-fixed cells but the cytoplasm often showed blebs, which were rarely found in Carnoy-fixed cells.

Finally, it was repeatedly observed that in smears of cells labeled with leucine, prior treatment with TCA before fixation resulted in a considerably lower autoradiographic background than when smears were first fixed and then extracted with TCA.

Effect of extractive procedures on the amount of radioactivity in cells labeled with tritiated precursors of nucleic acids. Carnoy-fixed smears from the previous experiment were used; sets of 3 smears each were treated as indicated in Table 23, which shows effects of such treatments on the intensity of the label. In cells labeled with H^3 -thymidine, only DNase produced a significant decrease in the intensity of the label. In cells labeled with cytidine, treatment with RNase or 10% PCA for 12 to 18 hr practically abolished the label, while 3 to 5 hr of treatment with PCA reduced it by 90%. DNase digestion also significantly decreased the intensity of the label in cells labeled with H^3 -cytidine. However, of the

solutions used for extractive procedures (Table 23), only the following were found to be radioactive: the 10% PCA and the RNase solutions used for smears of cytidine-labeled cells, and the DNase solution used for smears of thymidine-labeled cells. Both the PCA used to extract thymidine-labeled cells and the DNase solution used to digest cytidine-labeled cells were without radioactivity.

TABLE 23

Effect of extractive procedures on the intensity of the label in autoradiograms of Ehrlich ascites tumor cells labeled with tritiated compounds*

Extractive procedure	Mean grain count per labeled cell in smears**		Radioactivity in solution used for smears†	
	Thymidine	Cytidine	Thymidine	Cytidine
None	31	27	-	-
2% PCA	31	28	0	0
10% PCA, 3 hr	33	4	-	-
10% PCA, 5 hr	32	2	0	0.8
10% PCA, 12 hr	33	0	0	1.3
10% PCA, 18 hr	32	0	-	-
RNase	32	0	0	2.0
DNase, 7 hr	6	20	1.1	0
DNase, 16 hr	4	14	1.9	0
2% PCA after DNase	-	-	0	0

*All smears were fixed in Carnoy fluid. Values for grain counts are means of 3 smears in which the mean grain count was determined on 50 labeled cells. Radioactivity values are expressed in $\mu\text{c}/\text{ml} \times 10^{-3}$. Solution samples were taken from a volume of 40 ml in which 4 slides had been processed.

**0: not above background level, 0.2 grains per 100 μ^2 .

†0: not above background level, $3.1 \times 10^{-6} \mu\text{c}/\text{ml}$ or less.

A morphological difference was noticeable between cells treated with RNase and those extracted with PCA: In the former the cytoplasm appeared vacuolated, while in those treated with PCA the cytoplasm stained deeply with eosin. Untreated Ehrlich ascites cells have a basophilic cytoplasm.

The problem of nuclear versus cytoplasmic label in smears of cells marked with tritiated leucine. In autoradiography with tritiated compounds, it has been customary to attribute to radioisotope incorporated into the nucleus those silver grains that are found directly above the nucleus itself, and to radioactivity in the cytoplasm itself those silver grains found above it.

This is essentially correct for thin sections of tissues, but in smears of cells, the nucleus is covered by a thin layer of cytoplasm, which registers very efficiently because of its proximity to the emulsion.(13,17) It is thus possible that in smears some of the grains found directly above the nucleus of cells may be attributable to radioactivity in the cytoplasm rather than in the nucleus. This hypothesis was tested using smears and sections prepared from aliquots of ascites tumor. The sections were prepared by soaking Gelfoam in an aliquot of Ehrlich ascites tumor labeled with tritiated leucine, embedding in paraffin, and cutting 3- μ sections. Intensity of label per cell, per nucleus and per cytoplasm were then compared in smears and sections (Table 24). The mean grain count per labeled cell was almost 30% lower in sections than in smears; most of the discrepancy was due to a decrease in the number of grains above the nucleus. Thus, while in smears, labeling was slightly more intense in the nucleus than in the cytoplasm, the reverse was true in sections, indicating that part of the so-called nuclear activity found in smears is actually attributable to the thin coat of cytoplasm interposed between the nucleus and the emulsion.

TABLE 24

Intensity of the label in the nucleus and cytoplasm of
Ehrlich ascites tumor cells labeled with tritiated leucine

Fixative	Preparation	Mean grain count		
		Cell	Nucleus	Cytoplasm
Methanol	Smear	62	35	27
Carnoy	Smear	61	37	24
Methanol	Paraffin section	42	20	22
Carnoy	Paraffin section	42	18	24

Discussion

The factors that influence intensity of labeling in autoradiograms of cells labeled with tritiated compounds can be divided into three main groups: 1) factors concerned with the uptake of the radioactive compound by the individual cell, 2) effects produced by the different procedures intervening between sampling of the labeled tissue and autoradiography, and 3) the autoradiographic procedure and subsequent manipulations, such as staining and trypsinization of the emulsion. Factors related to the uptake of the radioisotope by the individual cell are still largely unknown and will not be considered here; it is sufficient to say that they are of little importance in determining differences in average intensity in smears or sections prepared from the same tissue sample. The effect

of the autoradiographic procedure and subsequent manipulations are better known and have been summarized in previous papers.(18,19) The experiments described in the present report deal exclusively with the effects on labeling intensity produced by various procedures intervening between sampling and autoradiography, such as fixation, histological preparation of the sample and histochemical procedures. Carnoy fluid and methanol were used here because they are the fixatives most frequently used in autoradiography(9,20,21) and for the enzymatic removal of nucleic acids.(8,22) Other alcohol mixtures are also very good, but, of the other most commonly used fixatives, Zenker is known to inactivate ribonuclease(23) and to desensitize the autoradiographic emulsion,(19) while Bouin's fluid or other formalin-containing fixatives make tissues more resistant to RNase and DNase treatment.(8,24) If nucleic acids are not to be removed from the tissues, formalin with or without TCA,(15) and Bouin's fluid(25) are, of course, excellent fixatives for autoradiographic purposes.

The results obtained in the present experiments indicated that both Carnoy and methanol were good fixatives and that they removed about the same amount of radioactivity from tissues for each of the three labeled precursors tested. After fixation in either Carnoy or methanol, extraction with 2% PCA or TCA had little effect on the labeling intensity, as previously observed by Feinendegen et al.(9) Treatment with TCA before fixation seemed to improve the quality of autoradiographs of cells labeled with tritiated leucine, by decreasing the background. It is possible that some TCA-soluble labeled material in the ascitic fluid may be precipitated by the fixative and become resistant to further treatment with TCA.

Our experiments with RNase confirmed previous results of other investigators indicating that under properly controlled conditions this enzyme specifically removes RNA.(8,22,23,26,27) Our finding that this enzyme removed practically all the radioactivity due to the incorporation of cytidine confirmed the observation of Feinendegen et al.(10) that in the first 2 hr after exposure to the radioisotope, more than 90% of the cytidine incorporated into nucleic acids is in the RNA fraction, while a considerable amount of radioactivity removable by fixatives is in the acid-soluble fraction. Leblond and Amano(11) similarly found that radioactivity from tissues of animals given an injection of H^3 -cytidine was completely removed by treatment with RNase. In Prescott's experiments(28) with cultures of protozoa and H^3 -cytidine, RNase removed 95-100% of the radioactivity incorporated in nucleus and cytoplasm. Feinendegen et al.(9,10) also found extraction with PCA a satisfactory method for the selective removal of RNA from cells. The specificity of PCA extraction, however, has been questioned by other authors.(29) Our results seem to indicate that extraction in 10% PCA in the cold for 12 to 18 hr gives results comparable to those obtained with RNase. Cells treated with PCA also looked better morphologically, in our opinion, than cells digested with RNase, and, considering that PCA extraction is a much simpler procedure than RNase digestion, we feel justified in suggesting PCA extraction as the method of choice for the removal of RNA.

Amano⁽⁸⁾ has reviewed the problem of digestion with DNase for the removal of DNA; as was reported by many others, our results with DNase were not so clear-cut as those obtained with RNase. In our material, although DNase markedly decreased radioactivity due to thymidine, it also decreased to a considerable degree the radioactivity due to cytidine. Curiously enough, however, no radioactivity was found in the DNase solution used to digest smears labeled with cytidine or in the PCA used to rinse these same smears. One could believe that the modifications induced by digestion with DNase may result in a desensitization of the emulsion.

The results also confirm the observation that in smears of cells labeled with radioactive leucine and perhaps with RNA precursors, grains found over the nucleus are not necessarily due to radioactivity incorporated into the nucleus.^(13,17) On the contrary, with leucine, almost half of the grains found over the nuclei of cells in smears are due to radioactivity present in the thin coat of cytoplasm above the nucleus.

References

1. Messier, B., and C. P. Leblond. Preparation of coated radioautographs by dipping sections in fluid emulsion. *Proc. Soc. Exptl. Biol. Med.* 96, 7-13 (1957).
2. Pelc, S. R. The stripping-film technique of autoradiography. *Intern. J. Appl. Radiation and Isotopes* 1, 172-177 (1956).
3. Herz, R. H. Photographic fundamentals of autoradiography. *Nucleonics* 9 (3), 24-39 (1951).
4. Baserga, R., and K. Nemeroff. Factors which affect efficiency of autoradiography with tritiated thymidine. *Stain Technol.* 37, 21-26 (1962).
5. Hughes, W. L., V. P. Bond, G. Brecher, E. P. Cronkite, R. Painter, H. Quastler, and F. G. Sherman. Cellular proliferation in the mouse as revealed by autoradiography with tritiated thymidine. *Proc. Natl. Acad. Sci.* 44, 476-483 (1958).
6. Edwards, J. L., A. L. Koch, P. Youcis, H. L. Freese, M. B. Laite, and J. T. Donalson. Some characteristics of DNA synthesis and the mitotic cycle in Ehrlich ascites tumor cells. *J. Biophys. Biochem. Cytol.* 7, 273-281 (1960).
7. Wimber, D., H. Quastler, O. L. Stein, and D. R. Wimber. Analysis of tritium incorporation into individual cells by autoradiography of squash preparations. *J. Biophys. Biochem. Cytol.* 8, 327-331 (1960).

8. Amano, M. Improved techniques for the enzymatic extraction of nucleic acids from tissue sections. *J. Histochem. Cytochem.* 10, 204-212 (1962).
9. Feinendegen, L. E., V. P. Bond, W. W. Shreeve, and R. B. Painter. RNA and DNA metabolism in human tissue culture cells studied with tritiated cytidine. *Exptl. Cell Research* 19, 443-459 (1960).
10. Feinendegen, L. E., V. P. Bond, and R. B. Painter. Studies on the interrelationships of RNA synthesis, DNA synthesis and precursor pool in human tissue culture cells studied with tritiated pyrimidine nucleosides. *Exptl. Cell Research* 22, 381-405 (1961).
11. Leblond, C. P., and M. Amano. Synthetic activity in the nucleolus as compared to that in the rest of the cell. *J. Histochem. Cytochem.* 10, 162-174 (1962).
12. Prescott, D. M., and M. A. Bender. Synthesis of RNA and protein during mitosis in mammalian tissue culture cells. *Exptl. Cell Research* 26, 260-268 (1962).
13. Taylor, J. H. Nucleic acid synthesis in relation to the cell division cycle. *Ann. N. Y. Acad. Sci.* 90, 409-421 (1960).
14. Woodard, J., E. Rasch, and H. Swift. Nucleic acid and protein metabolism during the mitotic cycle in *Vicia faba*. *J. Biophys. Biochem. Cytol.* 9, 445-462 (1961).
15. Mueller, H. G., and A. Linnartz-Niklas. Autoradiographische Untersuchung Über die Grösse der Eiweiss-synthese der weiblichen Genitalorgane im Metoestrus bei Ratte und Maus. *Arch. Gynaekol.* 194, 48-62 (1960).
16. Joftes, L. D. Liquid emulsion autoradiography with tritium. *Lab. Invest.* 8, 131-138 (1959).
17. Perry, R. P., M. Errera, A. Hell, and H. Durwald. Kinetics of nucleoside incorporation into nuclear and cytoplasmic RNA. *J. Biophys. Biochem. Cytol.* 11, 1-14 (1961).
18. Kisielewski, W. E., R. Baserga, and J. Vaupotic. The correlation of autoradiographic grain counts and tritium concentration in tissue sections containing tritiated thymidine. *Radiation Res.* 15, 341-348 (1961).
19. Kopriwa, B. M. and C. P. Leblond. Improvements in the coating technique of radioautography. *J. Histochem. Cytochem.* 10, 269-284 (1962).

20. Bogoroch, R., and B. V. Siegel. Some metabolic properties of the nucleolus as demonstrated by recent radioisotope experiments. *Acta Anat.* 45, 265-287 (1961).
21. Painter, R. Nucleic acid metabolism in HeLa S3 cells after X-ray induced mitotic delay. *Radiation Res.* 13, 726-736 (1960).
22. Swift, H. Cytochemical techniques for nucleic acids, in *The Nucleic Acids* (E. Chargaff and J. N. Davidson, editors), Vol. 2, New York, Academic Press, 1955. pp. 51-92.
23. Stowell, R. E., and A. Zorzoli. The action of RNase on fixed tissues. *Stain Technol.* 22, 51-61 (1947).
24. Stowell, R. E. The specificity of the Feulgen reaction for thymonucleic acid. *Stain Technol.* 21, 137-148 (1946).
25. Carneiro, J., and C. P. Leblond. Continuous protein synthesis in nuclei, shown by radioautography with H^3 -labeled amino acids. *Science* 129, 391-392 (1959).
26. Brachet, J., and J. R. Shaver. The effect of nuclease on cytochemical reactions for amino acids and on staining with acid dyes. *Stain Technol.* 23, 177-184 (1948).
27. Kurnick, N. B. Pyronine Y in the methyl-green pyronine histological stain. *Stain Technol.* 30, 213-230 (1955).
28. Prescott, D. M. Nucleic acid and protein metabolism in the macro-nuclei of two ciliated protozoa. *J. Histochem. Cytochem.* 10, 145-153 (1962).
29. Pearse, A. G. E. *Histochemistry*. Little, Brown and Co., Boston, 1961. p. 212.

SOME COMMENTS CONCERNING THE FEASIBILITY OF DETERMINING THE RADIATION-INDUCED MUTATION RATE FOR SEX-LINKED RECESSIVE LETHALS IN THE MOUSE

Douglas Grahn and Katherine F. Hamilton

In recent years, knowledge of the sex chromosomes of the mouse has vastly increased and the number of identifiable sex-linked mutations has steadily risen. A new one, sex-linked anemia, was very recently reported and mapped.⁽¹⁾ This new mutation was detected in the course of a brief effort to determine the radiation-induced rate of mutation for sex-linked lethals.⁽²⁾ The effort was not particularly successful, though only several hundred chromosomes were tested. In Auerbach's study, the irradiated x-chromosome was marked with the dominant gene Tabby (Ta) and the absence of this marker in the offspring of daughters of the original irradiated male was considered evidence for the presence of an induced lethal. The tested daughter also carried two dominant markers, Bent-tail (Bn) and Brindled (Mo^{br}), on the unirradiated x-chromosome to facilitate mapping of any observed lethal and to limit the chromosome region under observation. The presence of Bn, however, prevented the use of sex-ratio as a diagnostic tool since Bn is only 40-50% viable in the hemizygous male.

An alternative approach to the one used by Auerbach and co-workers would be to use sex-ratio as a criterion. This would employ one allele of Mo^{br} known as "Tortoise" (To), a recessive lethal with a dominant coat color expression in the carrier female. This mutation, or one of its alleles, has appeared spontaneously in several different laboratories and occurred here at Argonne National Laboratory in 1958. It arose in the C57BL/6 strain and is still carried in a non-sibmated stock derived from this strain. It is apparently completely penetrant though there is considerable variation in expression of the tortoise-shell, or mottled, coat color in the female. It is completely lethal in the male. A carrier female, To/+, will thus produce offspring with a nearly 2:1 sex-ratio. If the wild-type chromosome should carry a recessive lethal closely linked to To, few or no males will appear among her offspring. The sex-ratio will vary between 100:1 or more and 2:1 depending upon the recombination frequency. The efficiency could be improved by the addition of a second marker, such as Bn, if there were no associated viability effect. Unfortunately, there are viability effects, and these would add to those contributed by the Tortoise mutation.

Before embarking on any full-scale test for sex-linked lethals with the sex-ratio method, it was considered desirable to determine the lifetime reproductive capabilities of the To/+ female. It was estimated that at least 50 classifiable offspring had to be produced to make the system statistically acceptable. There is also the method of sequential analysis which could shorten the test period by discarding the presumed negative as soon as an

appropriate number of males appeared. While the details have not yet been worked out, it would be possible to discard some F_1 females as negative on the basis of only one or two litters.

Hybrid females appear to offer the best chance of obtaining adequate numbers, therefore $To/+$ (C57BL) females were mated to $+/-$ males of the BALB/c and A/Jax strains. $To/+$ F_1 females were then backcrossed to C57BL males for the lifetime performance tests. The F_1 females are listed as the BAF_1 (A/Jax male parent) and the BCF_1 (BALB/c male parent). Both F_1 females qualify for the test; litter sizes average 6.3, and 8 or more litters are readily produced. The data are summarized in Table 25.

TABLE 25

Summary of reproductive performance data on $To/+$ females

Strain	No of matings	No litters	Average litter size	Total born	Dead (unclassified) 0-5 days	Females						Males		Ratio	
						$To/+$ born	$To/+$ weaned	Dead	$+/-$ born	$+/-$ weaned	Dead	Total born	No weaned	σ/ϕ	$To/+$
$BAF_1 To/+$	14	120	6.3	464	27	195	166	15	242	242	0	280	266	5	1.7:1
$BCF_1 To/+$	13	120	6.3	471	22	216	157	24	236	236	1	274	253	7	1.7:1
$BAF_1 +/-$	5	64	9.2	29	10	-	-	-	24	28	3	28	277	3	10:1
$BCF_1 +/-$	5	25	10.2	125	4	-	-	-	125	121	3	121	125	4	10:1
C57BL $To/+$	12	84	4.1	200	66	66	46	30	66	60	11	126	97	29	1.6:1

Unfortunately, there is a definite effect on viability of the heterozygous female. Classification of To was done at about 8 days of age, and between that age and weaning age (ca. 30 days), 15% of the To females died in the BAF_1 and nearly 25% in the BCF_1 . Losses were negligible among $+/-$ females, and less than 10% of the males died before weaning. Sex-ratio for both hybrids was 1.7:1 (σ/ϕ) based on number born. The deficiency of females may be partly due to an intra-uterine loss of To heterozygotes since the control matings indicate a nearly perfect 1:1 sex-ratio.

Twenty-five test matings have produced six or more litters and from 30 to 80 offspring. Sex-ratios vary with the mating from 1.2:1 to 2.6:1. This, too, is an encouraging finding since a limited range of variation in sex-ratio around the expected 2:1 enhances the diagnostic value of the parameter. A sex-linked lethal that is 50 or more cross-over units from To , and that therefore appears to segregate independently of To , will produce a sex-ratio of 4:1. This is the minimum deviation from the 2:1 expectation; all other lethals with recombination values of less than 50% will produce sex-ratios between 4:1 and 100:1 or more for loci closely linked to To . The observation of a sex-ratio of 4:1 or more is, therefore, presumptive of the presence of an induced sex-linked lethal. The reported reproductive performance data suggest that this ratio will probably not occur as a false positive with great frequency.

A more definitive analysis of the reproductive performance data will be made when the present test matings and their controls have all completed their reproductive activity. At present only three BAF_1 To/+ females, four BCF_1 To/+ females and one BAF_1 +/+ female have been retired. A number of points will be examined - e.g., viability of To/+ vs. +/+ females in the neonatal and infant period, sex-ratio vs. litter size, distributional properties of the sex-ratio parameter and, if feasible, a photodensitometric measure of the portion of the pelage that is expressing the To gene. This last point is essentially an examination of the randomness of x-chromosome inactivation in the somatic tissues.

References

1. Falconer, D. S. and J. H. Isaacson. The genetics of sex-linked anemia in the mouse. Genet. Res. 3, 248-250 (1962).
2. Auerbach, C., D. S. Falconer and J. H. Isaacson. Test for sex-linked lethals in irradiated mice. Genet. Res. 3, 444-447 (1962).

PROGRESS REPORT: PLUTONIUM REMOVAL, TUMOR INCIDENCE STUDIES. IV.

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

In preceding reports of this series, survival and bone tumor incidence in mice following the injection of a polymeric form of plutonium were correlated with the degree of removal of skeletally deposited plutonium by therapy with diethylenetriaminepentaacetic acid (DTPA), initiated 3 days after plutonium administration.^(1,2) A parallel experiment, using a monomeric form of plutonium, was initiated to compare tumor incidence following the two forms of plutonium, with and without DTPA therapy, and also to determine the effect of immediate therapy (1 hr). This report describes the effects of DTPA therapy begun after 1 hr and after 3 days on the distribution and excretion of this monomeric plutonium and summarizes preliminary survival and tumor incidence data.

The experimental methods paralleled those in the first experiment.⁽²⁾ CF#1/Anl female mice about 75 days old and having a mean weight of 24.3 g, were injected intravenously with about 2.7 μ c of Pu²³⁹/kg, in 0.30 ml. The plutonium was prepared from a concentrated nitric acid solution by dilution with 1% sodium citrate and adjustment to pH 6.2. The

TABLE 26

Experimental design for survival and tumor incidence studies on mice receiving monomeric plutonium

Treatment	Time initiated*	No. of mice
DTPA	1 hr	53
DTPA	3 days	51
Saline	1 hr	24
Saline	3 days	27

*Treatment was given once per day for 12 days.

plutonium at injection was 93% ultrafilterable* through Visking casing and was therefore considered to be monomeric. An intraperitoneal injection of 500 mg/kg of DTPA, prepared as before,⁽²⁾ or an equivalent volume of saline solution, was given either 1 hr or 3 days after the plutonium injection and once per day for 12 days. A total of 155 plutonium-injected mice were divided into 4 groups for study of tumor incidence and survival (Table 26). An additional 102 mice received only DTPA as controls for possible long-term effects of the therapy. All long-term mice were sacrificed with Nembutal when mor-

ibund, necropsied and subjected to skeletal roentgenography. A group of 180 mice that had received plutonium and either DTPA or saline were sacrificed at intervals for plutonium analysis. The collection of excreta and the plutonium analyses were performed as described previously.⁽⁴⁾

*Solutions of this filterability have subsequently been shown to be 100% dialyzable.⁽³⁾

Results and Discussion

Distribution and excretion of plutonium injected in monomeric form. In controls, the plutonium was about equally divided between bone and liver at the time when uptake was at a maximum (between 1 and 6 days after injection) (Table 27). The plutonium level in spleen was low (0.2% of injected dose at 3 days). That in liver diminished rapidly so that only about one-tenth of the initial amount deposited in the liver was retained 30 days later. This pattern of distribution and retention indicates that the plutonium in these tissues was most likely not located within the reticuloendothelial cells, and it can be concluded that this monomeric solution did not become significantly polymerized after its injection.⁽⁴⁾ The level of plutonium in bone at 6 days remained constant for 90 days. However, it had dropped about one-third by 300 days.

TABLE 27

Effect of 12 days of DTPA therapy on distribution and retention of plutonium injected in monomeric form

Time after Pu injection	Plutonium content, % injected dose*					
	Bone**			Liver		
	Saline controls	DTPA at 3 days	DTPA at 1 hr	Saline controls	DTPA at 3 days	DTPA at 1 hr
1 hr	15.2 ± 0.5	-	-	28.6 ± 1.6	-	-
1 day	24.8 ± 1.6	-	12.2 ± 0.5	35.9 ± 1.5	-	4.7 ± 0.5
3 days	24.5 ± 1.2	-	9.9 ± 0.3	32.8 ± 1.3	-	0.7 ± 0.07
6 days	29.7 ± 1.3	23.0 ± 1.3	8.6 ± 0.5	23.3 ± 1.3	2.3 ± 0.3	0.3 ± 0.04
12 days	29.6 ± 2.8	19.0 ± 1.1	7.0 ± 0.3	12.7 ± 0.6	0.4 ± 0.05	0.1 ± 0.01
15 days	30.7 ± 2.3	14.0 ± 1.0	-	9.4 ± 1.0	0.3 ± 0.02	-
30 days	31.0 ± 0.5	-	-	5.2 ± 0.8	-	-
90 days	29.7 ± 0.9	16.9 ± 2.1	6.3 ± 0.3	1.9 ± 0.1	0.6 ± 0.1	0.2 ± 0.02
300 days	20.8 ± 1.7	12.7 ± 0.4	5.1 ± 0.5	1.2 ± 0.02	0.6 ± 0.2	0.2 ± 0.01

*All values shown are the mean of 3 samples, each containing the pooled tissues from 2 mice, ± the standard error of the mean.

**The bone content is calculated from mean femur content x 20.

When DTPA therapy was begun 3 days after plutonium injection, loss of plutonium from liver was accelerated, so that almost all was removed after 3 days of therapy, and the level in bone was reduced by about one-half after 12 days (Table 27). When initiated 1 hr after plutonium, the same course of therapy again removed one-half of the deposited plutonium from bone and nearly all from liver. In addition, it prevented all deposition in bone after 1 hr, so that the total retention in bone in these treated mice was less than one-quarter of that of the saline controls.

Like the controls, both treated groups lost plutonium from the bone between 90 and 300 days; however, the proportion lost in the treated groups was somewhat smaller, indicating that less of the retained plutonium was available to the naturally occurring complexing agents present in the body.

The excretion of plutonium in the control and treated groups is shown in Table 28. As found previously,⁽⁴⁾ the amount of plutonium lost from the liver is the same as that in the feces, and that removed from bone is equal to the amount appearing in the urine. The data also show that 12 days of therapy was sufficient and that further consecutive daily injections would have promoted little or no increased effect.

TABLE 28

Effect of DTPA on cumulative excretion of plutonium injected in monomeric form

Time after Pu	Plutonium content, % injected dose*					
	Feces			Urine		
	Controls	DTPA at 3 days	DTPA at 1 hr	Controls	DTPA at 3 days	DTPA at 1 hr
1 day	5.6	-	25.2	10.1	-	25.1
3 days	8.1	-	34.8	10.6	-	27.2
6 days	13.4	36.2	37.8	10.8	16.0	27.9
12 days	25.4	40.1	39.2	11.1	18.3	28.8
15 days	28.2	41.0	-	11.1	18.6	-

*All values represent the mean of two samples, each containing the pooled excreta from 4 mice.

Tumor incidence and survival. At the present time only a preliminary survey of bone tumor incidence following administration of monomeric plutonium can be made since many of the mice are still living and only roentgenographic criteria are as yet available for the diagnosis of tumors. The criterion for tumor incidence now used is the presence of a positive or a probable tumor on the roentgenogram (right side of Table 29). Positive tumors are defined as those obviously outside of the usual bone boundary. Probable tumors are defined arbitrarily on the basis of roentgenographic abnormalities which have, in an earlier experiment, proved to be osteogenic sarcomas on histological examination. The use of these two categories provides an index of tumor incidence roughly equivalent to the more refined index, based on histological examination of selected bone

areas, that was used in the experiment with polymeric Pu (left side of Table 29 and Reference 2); thus a preliminary comparison can be made between the two experiments.

TABLE 29

Effect of DTPA at 3 days on bone tumor incidence and survival up to 400 days in mice injected with two forms of plutonium

	Polymeric Pu, 2.6 $\mu\text{c}/\text{kg}$		Monomeric Pu, 2.7 $\mu\text{c}/\text{kg}$	
	Saline	DTPA	Saline	DTPA
No. of mice	104	105	51	51
400-day survival, %	54	65	28	65
Latent period*	269	325	153	236 [†]
Tumor incidence, %**	56	25	81 [†]	67 [†]

*Days to death of first mouse with a tumor.

**Proportion of mice dying that had at least one bone tumor at death within the period from the end of the latent period to 400 days.

[†]Preliminary.

The data summarized in Table 29 suggest that after administration of monomeric plutonium, bone tumors appeared earlier, i.e., the latent period was shorter, and there was a higher proportion of mice with a bone tumor at death than after administration of the same dose of polymeric plutonium. These differences must in part be related to the 1.5 times greater long-term skeletal retention of plutonium following injection of the monomeric form.⁽⁴⁾ The preliminary results also suggest that the removal of half of the monomeric plutonium from bone by DTPA therapy at 3 days is not followed by a 50% reduction in tumor incidence, in contrast to the case in the experiment with polymeric plutonium.

At 400 days the group of mice injected with monomeric plutonium and treated with DTPA at 1 hr had about the same survival (85%) as that of the control group receiving only DTPA (90%). In addition, the latent period was longer and tumor incidence was lower in this early-treated group than in the 3-day treated group; i.e., the only mouse that died with a possible bone tumor, one requiring histological diagnosis, did not die until the 354th day.

References

1. Rosenthal, M. W., J. F. Markley, J. Schubert, and A. Lindenbaum. Progress report: Plutonium removal, tumor incidence studies. III. Biological and Medical Research Division Semiannual Report, January through June 1962, Argonne National Laboratory. ANL-6723, pp. 144-145.
2. Rosenthal, M. W., J. F. Markley, A. Lindenbaum, and J. Schubert. Influence of DTPA therapy on long-term effects of retained plutonium. Health Phys. 8, 741-745 (1962).
3. Lindenbaum, A. and W. M. Westfall. Unpublished information.
4. Schubert, J., J. F. Fried, M. W. Rosenthal, and A. Lindenbaum. Tissue distribution of monomeric and polymeric plutonium as modified by a chelating agent. Radiation Res. 15, 220-226 (1961).

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

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

In the estimation of radiation dose absorbed by bone after skeletal deposition of a bone-seeking radioelement, it is essential to distinguish quantitatively between the radioelement located in the bone itself and that deposited in the marrow. This distinction is especially important in the case of plutonium, both because of the short range of the α radiation relative to sensitive areas of bone and marrow, and because its uptake in marrow may vary considerably, depending on the physical state of the plutonium administered. Neither the measurement of total radioactivity in the bone nor the analysis of skeletal autoradiographs can give the necessary quantitative measurement of plutonium in bone and bone marrow. Complete physical removal of marrow is, in many cases, impractical.

A chemical technique using the protein solvent Hyamine* [p-(diisobutylcresoxyethoxyethyl) dimethylbenzylammonium hydroxide] has been developed by which all exposed organic material, including marrow, can be dissolved from bone. The work reported here demonstrates that this removal of the organic portion of the bone with its associated plutonium does not remove the radioelement that is deposited in the hard bone.

Mice and rats were sacrificed 3 days after intravenous injection of about 2.7 and 1.6 $\mu\text{C}/\text{kg}$ of Pu^{239} , respectively, as plutonium citrate. Both ends of the tibia were cut off to obtain a segment of shaft of as uniform length and weight as possible and containing little or no trabecular bone. From each animal one weighed tibial segment was used for removal of marrow by Hyamine, the other for removal by physical means. Removal by Hyamine was achieved by covering the cracked or cut bone with two or three changes of the solvent over a period of 2 weeks, until the solution no longer became colored, and the marrow appeared to be completely removed. The Hyamine was then drawn off, and the bone was rinsed several times with 95% ethanol, dried with acetone, and then wet-ashed with concentrated HNO_3 and prepared for plutonium analysis. Physical separation was achieved by irrigation of the marrow canal with saline solution. Since Hyamine solutions cannot be easily ashed, the plutonium extracted by the Hyamine must be measured indirectly by subtracting the amount of radioactivity in a bone sample treated with Hyamine from that

---

*Hyamine 10-X, obtained from Packard Instrument Company,
LaGrange, Ill.

in a comparable untreated sample, either from the same animal or from another of the same experimental group.

The results with Hyamine extraction did not differ significantly from those with saline irrigation (Table 30). Thus it appears that the Hyamine extraction did not remove an appreciable amount of the plutonium attached to endosteal and periosteal surfaces of the shaft segment. This technique should therefore be valuable in the quantitative differentiation between the plutonium in marrow and that in cortical bone or on trabecular and other bone surfaces, especially in skeletal areas such as the spine, in which physical removal of the marrow is not possible.

TABLE 30

Pu^{239} content of tibial shaft segments after removal of marrow by saline irrigation or Hyamine extraction

Species	No. of animals*	% injected Pu in marrow-free shaft per g wet weight of intact shaft, mean $\pm$ S.E.	
		Saline irrigation	Hyamine extraction
Mouse	3	12.0	13.9
	3	11.2	11.5
	3	9.4	11.0
	2	11.6	11.9
		11.05 ± 0.57	12.08 ± 0.64
Rat	2	0.63	0.55
	2	0.72	0.80
	2	0.69	0.64
	2	0.63	0.57
		0.668 ± 0.023	0.638 ± 0.057

*Each animal in a comparison contributed the shaft segment from one tibia to a pooled sample for physical removal of marrow, the other tibia to a pooled sample for Hyamine extraction.

The applicability of the technique is currently being studied in a comparison of the partition of colloidal and ionic forms of plutonium between marrow and hard bone in the whole mouse skeleton. The results of this study will be valuable in correlating deposition sites of plutonium in bone with bone tumor induction by the radioelement.

MUCOPOLYSACCHARIDES AND MUCOPROTEINS OF OSSIFYING CARTILAGE

Arthur Lindenbaum

Abstract

It is evident that rapid physical and biochemical transformations must take place when cartilage is transformed into bone. Calf scapula has been found to be ideal material in which to study these processes. With the ossification front as a guide, parallel cuts of the cleaned scapulas yielded three portions designated as resting cartilage, transforming cartilage, and new bone. The bone was decalcified by dialysis against 0.5 N acetic acid. Each portion was then acetone-dried, hydrolyzed enzymatically, deproteinized with 3:1 chloroform-amyl alcohol, and fractionated with ethanol to precipitate mucopolysaccharides (MPS). Only chondroitin sulfates A and C were found in significant amounts. Total yields of MPS were 24% for both cartilage zones and 6.5% for new bone on the basis of dry organic material. After repeated enzymatic hydrolysis, deproteinization, and ethanol fractionation, MPS fractions were still found to contain as much as 3-5% amino acid residues, among which serine and glutamic acid were found in high proportion. Amino acid analysis of a typical MPS fraction indicated a composition distinct from collagen and containing several unknowns. Mucoproteins extracted from resting and transforming cartilage were analyzed with respect to calcium, total protein, amino acids, etc., preliminary to the study of the affinity of these native substances for bone-seeking cations.

MUCOPROTEIN EXTRACTS OF OSSIFYING CARTILAGE

Klaus E. Kuettner and
Arthur Lindenbaum

Abstract

To investigate the cation-binding properties of ossifying cartilage it is first necessary to obtain purified components of this tissue in an undegraded state. Several approaches to the extraction of native mucoproteins have been attempted, using calf scapula cartilage. These consist of water extraction of high-speed* homogenates for varying times (1 hr to 9 days) at 4°C and, in addition, decalcification with cation exchangers and ultrasonics. Freshly extracted, as well as stored solutions, were subjected to physical and chemical analysis. The absence of collagen in the water extracts was demonstrated by amino acid analysis. All extracts contained detectable amounts of glucosamine although no glucosamine-containing mucopolysaccharide has so far been demonstrated in this tissue. Ultracentrifuged fresh extracts appear to consist mainly of a single component with a sedimentation coefficient of 11-15S and a molecular weight of about 1.5 million. Decalcification of fresh extracts yielded a mucoprotein of reduced viscosity and molecular weight. These results indicate the feasibility of quickly obtaining an essentially undegraded native mucoprotein from ossifying cartilage.

---

*Homogenized at 45,000 rpm in a VirTis "45" homogenizer.

GOLGI FINE STRUCTURE IN A RADIOSENSITIVE GIANT AMOEBA

Edward W. Daniels and Nancy Reed

Amoebae of the radiosensitive species, Pelomyxa illinoisensis, were fixed in OsO_4 during interphase and in various stages of mitosis. Thin sections from Epon blocks containing single organisms were studied with an RCA EMU 3F electron microscope. Randomly distributed Golgi bodies were found, many of which resemble small pyramids or cones somewhat smaller than the adjacent mitochondria. There were flattened vesicles at the base and in the midportion of each Golgi and rounded vesicles at the apex. A number of the latter have an internal fringe similar to that on the plasmalemma and on the interior surface of fresh pinocytosis vesicles that originally come from the plasmalemma. Partly flattened, club-shaped vesicles have been found in some Golgi bodies and also singly in the cytoplasm. These may represent intermediate stages of flattening between the apical and basal components. The flattened vesicles of a Golgi body often display small lateral blebs which occasionally contain minute electron-dense particles. It is our present interpretation that pinocytosis vesicles, and possibly other small vesicles, collect at the apex of the Golgi body and gradually become integrated and flattened. The formation and fate of Golgi bodies in these amoebae is being studied by electron microscope methods during the feeding and starvation cycle, and at different mitotic stages. Pairs of Golgi bodies were observed in relatively high frequency in some amoebae during mitosis. The frequency and significance of this is being investigated further.

EXTRACTION OF DNA AND RNA FROM RADIOSENSITIVE AMOEBAE AND THE USE OF THESE COMPOUNDS IN RADIORESTORATION AND TRANSPLANTATION EXPERIMENTS

Edward W. Daniels, Evelyn P. Brown,
and Anna K. Laird

Unirradiated, living protoplasm is a highly potent therapeutic agent when injected into supralethally irradiated amoebae of the same species (Pelomyxa illinoisensis),^(1,2) and we are investigating the possibility that DNA or RNA may be the active component. In previous experiments⁽³⁾ commercial DNA and RNA from mammalian sources were injected into lethally irradiated Pelomyxa illinoisensis amoebae but failed to bring about recovery. This report presents the results of similar experiments with concentrated DNA and/or RNA from unirradiated donor amoebae. DNA and RNA prepared from milliliter quantities of unirradiated donors were administered either by injection into supralethally irradiated (X-rays) amoebae or by washing the amoebae in solutions of the compounds. However, no organisms recovered.

Finally, we investigated the possibility that DNA or RNA from Pelomyxa illinoisensis injected into Pelomyxa carolinensis might alter the heterologous transplantation tolerance.⁽⁴⁾

Methods

DNA and RNA were extracted from unirradiated amoebae of the species Pelomyxa illinoisensis by the phenol method of Kirby,^(5,6) with various modifications.^(7,8) Nucleic acids were freshly prepared for each experiment. An abbreviated description of our procedure follows:

One to three ml of donor amoebae were washed and packed in a centrifuge tube, and were then homogenized, either in 6% sodium para-aminosalicylate containing versene⁽⁷⁾ for extraction of DNA, or in water or duponol⁽⁸⁾ for extraction of RNA. The homogenate in each case was mixed with an equal volume of 90% phenol in water; an emulsion was formed by stirring at 0°C for 1 hour for the preparation of DNA, or by shaking at 60°C for 3 minutes for the preparation of RNA. The phenol-emulsion was broken by centrifugation at approximately 15,000 x g. The aqueous top layer, which contained the nucleic acid, was pipetted off. The nucleic acid was then precipitated with alcohol, redissolved in distilled water, and purified by reprecipitation. The final preparation was dissolved in distilled water for injection; the concentration of RNA was 300-500 μ g per ml; that of the DNA was not determined because of the very small quantity obtained.

Such preparations of RNA and DNA contain no protein precipitable by TCA; the RNA solutions are contaminated by an unidentified carbohydrate which gives a positive anthrone test. Extraction in duponol yields approximately three times as much RNA as in water, when both extractions are carried out at 60°C; the quantity of RNA extracted at 0°C is very small.

After DNA and RNA extraction the microinjections were done in a 5°C cold room where all equipment and solutions were pre-cooled.

Recipient amoebae were irradiated with 30 kr of X-rays, which is more than twice the lethal dose. Irradiated amoebae were washed and brought into the cold room for injection in one to 20 hours after exposure. The volumes of concentrated DNA and/or RNA injected were equivalent to about 25% of the volume of the recipient amoeba. Unirradiated amoebae were injected for controls. DNA, RNA, or mixtures of the two diffused freely throughout the protoplasm and mingled with the granular components of the cytoplasm.

Each injected amoeba was isolated immediately in a glass cup containing a milliliter or less of glass-distilled water and food organisms similar to those used for the stock amoeba cultures (paramecia and flagellates).

For the bathing procedure the X-irradiated amoebae were isolated into similar cups with varying dilutions of nucleic acids ranging from 1:1 to 1:100 in water. All of the experimental and control cultures as well as stock amoebae were kept in a constant temperature room set at 21°C. Daily observations were made until all of the amoebae died.

Results

In the first experiment, we injected DNA solution by micropipette into 30 X-irradiated amoebae. All of those injected died within 9 days, most of them within the last half of this period, as did the 100 irradiated controls (Table 31). There was no significant difference in survival time between the two irradiated groups, and there were few or no deaths among the 100 + unirradiated controls.

In the second experiment RNA was injected into 65 irradiated amoebae; again all died within 9 days. The irradiated controls (about 200) died within the same period while the 200 unirradiated controls survived and reproduced. We repeated this entire experiment twice more with similar results.

TABLE 31
Summary of RNA and DNA experiments

Recipient species	Treatment	Means of administration	No. of amoebae	No. of survivors at 9 days
<u>Radiorestoration experiments</u>				
<u>P. illinoisensis</u>	X-ray + DNA	injection bath	30	0
	X-ray + DNA		114	0
	X-ray		250	0
	Control		100	>100*
<u>P. illinoisensis</u>	X-ray + RNA	injection	65	0
	X-ray		200	0
	Control		200	>200
<u>P. illinoisensis</u>	X-ray + RNA	injection	26	0
	X-ray		100	0
	Control		100	>100
	X-ray + RNA	bath	20	0
	X-ray + RNA		30	0
	X-ray + RNA + DNA	injection	6	0
	X-ray + RNA + DNA	injection	30	0
	X-ray		150	0
	Control		100	>100
<u>Transplantation tolerance experiments</u>				
<u>P. illinoisensis</u>	X-ray + RNA-injected <u>P. carolinensis</u>	fusion	22	0
	X-ray + DNA-injected <u>P. carolinensis</u>	fusion	28	0
<u>P. carolinensis</u>	RNA from <u>P. illinoisensis</u>	injection	29	>29
	DNA from <u>P. illinoisensis</u>	injection	28	>28

Survivors for control groups were not scored individually; * indicates that the groups survived and reproduced.

Two groups of 20 and 30 irradiated amoebae were bathed in the RNA solutions for approximately 1 hr while they were observed with a dissecting microscope. Two other groups of 6 and 20 irradiated amoebae were injected with a 1:1 mixture of the RNA and DNA solutions. All were then washed and cultured for the remainder of their lives, i.e., several days. Again, these amoebae and their 50 and 100 irradiated controls all died, while the 100 unirradiated survived.

Finally, we investigated the effects of DNA and RNA on growth and heterologous transplantation tolerance in the amoebae. Pelomyxa carolinensis amoebae were injected with RNA or DNA from Pelomyxa illinoisensis and observed for several days; all divided and appeared to be in good condition. Amoebae from these cultures were then fused with Pelomyxa illinoisensis to determine whether or not tolerance was altered; all of the 22 RNA-injected and the 28 DNA-injected amoebae so tested died within 9 days. Although we have not analyzed the data statistically, the average time of death appears to be about the same as that of similar fusions without prior injection of Pelomyxa carolinensis with RNA or DNA.⁽⁴⁾

Pelomyxa carolinensis were found to grow and multiply after the injection of either DNA or RNA from Pelomyxa illinoisensis. Observations for 3 weeks after injection disclosed no differences from uninjected controls in morphology or functional activity.

References

1. Daniels, E. W. Micrurgical studies on irradiated pelomyxa. Ann. N. Y. Acad. Sci. 78, 662-674 (1959).
2. Daniels, E. W., and H. H. Vogel, Jr. Effects of nonirradiated protoplasm on recovery of amoebae exposed to supralethal doses of fission neutrons. Radiation Res. 10, 584-596 (1959).
3. Daniels, E. W. Microinjection studies on amoebae after their exposure to a supralethal dose of X-rays or γ -rays. Biological and Medical Research Division Semiannual Report, January through June 1959, Argonne National Laboratory. ANL-6200, pp. 82-88.
4. Daniels, E. W. Limits of transplantation tolerance in large amoebae. I. Microfusion studies using Amoeba proteus, Pelomyxa illinoisensis, and three strains of Pelomyxa carolinensis. J. Protozool. 9, 183-187 (1962).
5. Kirby, K. S. A new method for the isolation of ribonucleic acids from mammalian tissues. Biochem. J. 64, 405-408 (1956).
6. Kirby, K. S. The preparation of deoxyribonucleic acids by the p-aminosalicylate-phenol method. Biochim. Biophys. Acta 36, 117-124 (1959).
7. Hays, E. F., and J. A. Carr. Oncogenic properties of deoxyribonucleic acid isolated from parotid gland tumors. Cancer Res. 22, 1319-1322 (1962).
8. Scherrer, K., and D. E. Darnell. Sedimentation characteristics of rapidly labeled RNA from HeLa cells. Biochem. Biophys. Res. Commun. 7, 486-490 (1962).

FRACTIONATION OF AMOEBAE IN VARIOUS MEDIA

Edward W. Daniels, Evelyn P. Brown, and Y. E. Rahman

The objective of this work is to obtain a fractionation medium for Pelomyxa illinoisensis and Pelomyxa carolinensis amoebae which will 1) preserve the normal ultrastructure of the subcellular fractions and 2) preserve the radiorestorative capacity of Pelomyxa illinoisensis protoplasm. Previous work has shown that microtransfer of protoplasm directly from an unirradiated donor amoeba into a supralethally X-irradiated amoeba prevents death and promotes permanent culture formation.⁽¹⁾ This work has been extended by the centrifugation of donor amoebae and the transfer of enriched strata into lethally irradiated recipients.^(2,3) We found that some unirradiated protoplasmic components are required for recovery, while others are not.⁽⁴⁾ However, none of these strata from centrifuged amoebae was composed of a single species of subcellular component.

Previous experiments on radiorestoration with fractionated homogenates of amoebae have been unsuccessful,⁽⁵⁾ in part because of the very small quantity of homogenate obtainable, and because the fractionation medium was unsuitable. We have recently developed culture methods for growing Pelomyxa illinoisensis amoebae in very much larger quantities, which will greatly aid the fractionation work. We are now trying to improve the quality of the fractionation medium. Preservation of the normal ultrastructures is important in this work since enzymatic activities of homogenates are more likely to reflect normal intracellular conditions if the ultrastructures can be preserved. We have achieved some success in preserving normal ultrastructures in fractionation media, and this work is being completed with an electron microscopic study of sucrose gradient fractions of Pelomyxa illinoisensis.

Methods

Culture methods. Pelomyxa carolinensis were cultured in glass-distilled water and kept at room temperature (ca. 25°C). They were fed on Tetrahymena or on Paramecium caudatum mixed with Chilomonas paramecium. The amoebae were starved for 4 days before homogenization to rid them of food vacuoles. Pelomyxa illinoisensis were cultured in glass-distilled water and kept in a constant-temperature room at 21°C. They were fed Paramecium caudatum and Chilomonas paramecium, but they did not adapt to Tetrahymena. They were starved for 3 to 4 days before homogenization.

Preparation of homogenates. The amoebae were collected, washed in glass-distilled water, concentrated, and washed in the chosen homogenization medium. They were again concentrated, their volume was measured,

and the supernatant was discarded. One milliliter of packed amoebae was used in each experiment. After the addition of 2 ml of ice-cold homogenization solution, the cells were dispersed and transferred to a glass homogenizer fitted with a glass pestle which broke the cells but left nuclei intact. An additional 2 ml of solution were added to make a final homogenate volume of 5 ml.

The homogenate was then centrifuged at very low speed ($50 \times g$) in the International centrifuge for 2 min. The sediment, which contains mostly cell debris, crystals, food vacuoles and some nuclei, was discarded, and only the remaining supernatant was used for density-gradient centrifugation and electron microscopic studies.

Sucrose density-gradient centrifugation procedure. One ml of the prepared amoebae homogenate was layered on top of a gradient tube. Each gradient tube was prepared by layering successively 2 ml of 30, 25, 20, 15, and 10 per cent sucrose into a 12-ml plastic centrifuge tube (6). The gradient tubes were stored in a cold room at about 4°C for 16 to 20 hr before use.

Centrifugations were performed in the SCR swinging rotor of the Lourdes centrifuge operated at 8,000 rpm ($9000 \times g$) for 15 min. The top 6-ml solutions of the gradient tube were then carefully isolated with a syringe and a specially bent needle. This first fraction (fraction No. 1) contains essentially only the vesicles. The next 3 ml (fraction No. 2) were collected under the same procedure, and this fraction consists essentially of mitochondria. The remaining 2 ml make up the third fraction (fraction No. 3), which contains about 90% of the nuclei and a few mitochondria.

Electron microscopy The cell fractions thus obtained were then centrifuged at $30,000 \times g$ for 45 min to one hr. The supernatant was discarded, and the pellets obtained were fixed for 30 min to 1 hr in a mixture containing 5 parts of 2% osmium tetroxide, 2 parts 0.1 N HCl, and 1 part 8.5% NaCl, buffered with 2 parts sodium veronal acetate (7). The ice-cold fixative was poured into the tube containing the pellet, and for better penetration the pellet was immediately broken up with a clean, sharpened stick. At the end of the fixation period all of the tubes were recentrifuged, decanted, and dehydrated in a graded series of ethanols. The pellets were broken after each step and the tubes centrifuged again. Propylene oxide was used as a clearing agent, and the sediments were finally embedded in Epon 812 resin. After hardening in 35°C , 45°C , then 60°C ovens, the blocks were sectioned with a Porter-Blum microtome and examined in an RCA EMU 3F electron microscope.

Results

Effect of the fractionation media

Toxicity for whole amoebae. Since we planned to inject the homogenate fractions into P. illinoisensis, we first tested the effects of the various media on these organisms by microinjection (Table 32). The least toxic media were dextrose and Ficoll;* following these, in order of increasing toxicity, were glycerol, raffinose, and Tween 80.

TABLE 32

Toxicity of various fractionation media injected into Pelomyxa illinoisensis

Injections were made in a 5°C cold room with precooled instruments and solutions. The pH of homogenized Pelomyxa illinoisensis protoplasm in a negligible quantity of neutral glass-distilled water is 6.65.

Fractionation media were adjusted to pH 6.5 to 7.3.

Medium	Concentration, %	Number of amoebae	Visual Effects and Cell Damage		Survival at 1 week, %
			Immediate (1st hr)	Latent (6-8 days)	
Albumin	6	10	Cell damage: amoebae stop feeding, etc.	Toxic	30
Dextrose	5	ca. 50	No observed damage at 100X magnification	Good	ca. 100
Ficoll	3	"	"	"	"
Glycerol	50	10	"	Toxic	50
Gum arabic	5	10	Not well tolerated, feeding stops, etc.	"	60
D-Mannose	5	10	Not as well tolerated as dextrose	Fair	70
Raffinose	5	16	Moderately well tolerated: better than albumin, gum arabic, and d-mannose but poorer than dextrose, Ficoll, and glycerol	Good	88
Sucrose	8.5*	50	Toxic	Toxic	
Tween 80	5	12	Moderately well tolerated (mixed with homogenate)	Fair	67

*0.25 M.

*Ficoll (Aktieselskabet Pharmacia) is a polymolecular neutral colloid with properties similar to the polysaccharides. The average molecular weight is about 50,000.

Phase microscopy of nuclei in homogenates. One criterion for judging the value of fractionation media is its effect on the structure of the nuclei in the homogenate. We therefore examined nuclei in fresh homogenates with a phase microscope. These data are presented in Table 33. The structural detail in the nuclei was lost immediately with dextrose, but remained quite normal in Ficoll at the 3% and 4% concentrations. Ficoll with dextrose was poorer than Ficoll alone, and glycerol with Ficoll did not improve the appearance of the nuclei. Glycerol in 50% to 60% concentrations preserved the size and structure of the nuclei very well. However, electron micrographs of nuclei in glycerol show that the nuclear envelope was broken in many places. PVP preserved the nuclear size and appearance for a short time, but the stability of the nuclei in this medium was poor. The addition of dextrose to PVP did not improve this. None of the other media shown in Table 33 was satisfactory although Tween 80 at 3% was better than the rest.

TABLE 33

Effects of various fractionation media on the diameters of *Pelomyxa illinoisensis* nuclei (5°C; pH 6.5 to 7)

Medium	Concentration, %	Number of nuclei measured	Average nuclear diameter, μ	Comments
Control, in vivo		47	20.7	Amoebae were flattened and nuclei measured; nucleoli were clearly visible.
Dextrose	5	20	22.9	Nuclear damage clearly apparent; nucleoli absent.
Ficoll	2.3	30	20.7	Nucleoli and nuclear granules fairly clear, but faded within 15 min.
Ficoll	2.4	20	21.2	Nucleoli clear, but faded after 5 min.
Ficoll	2.5	50	19.4	Nucleoli very clear; damage minimal.
Ficoll	3	50	20	Nuclei quite normal, nucleoli and granules clear for 15 min.
Ficoll	4	50	18.9	Nuclei as good or better than 3% Ficoll after 15 min; granular but not as good as control nuclei.
Ficoll	5	50	19.2	Granulation and nucleoli clear for 15 min. After 20 min some central clearing in the nuclei. After 2 hr nuclei badly faded, many burst.
Ficoll + Dextrose	2.5 5	70	18.7	Many nuclei burst; nucleoli rarely seen.
Ficoll + Glycerol	0.83 50	13	18.2	No improvement over 50% glycerol or Ficoll.

TABLE 33 (Contd.)

Medium	Concentration, %	Number of nuclei measured	Average nuclear diameter, μ	Comments
Glycerol	25	7	18.9	Nuclei damaged.
Glycerol	40	-	-	Nuclei inferior to those in 50% glycerol; nucleoli gradually disappear within 10 min.
Glycerol	45	30	28.2	Nuclei better than in 40% glycerol; nucleoli faded considerably within 15 min at 6°C.
Glycerol	50	50	19.1	Nucleoli excellent; nuclei good for at least 15 min.
Glycerol	55	30	21	Nuclei fairly good; peripheral nucleoli quite distinct; center of nucleus somewhat washed out; stability fair after 10 to 15 min; occasional fading of granular structures; very few nuclei burst.
Glycerol	60	50	19.6	Nuclei very good after 10 to 15 min; nucleoli good; center of nucleus good (granular); nuclei essentially unchanged after 1 hr, but slight fading.
Glycerol	75	-	-	Nuclei shrunk and damaged.
PVP*	3	30	20	Nuclei quite good, granulated with nucleoli; most nucleoli granular, not solid as in normal cells; after 10 to 15 min, nuclei somewhat washed out, with nucleoli and granules fading; many nuclei burst.
PVP	4	-	-	Nuclei fair to poor; not as good as 3% PVP.
PVP	6	20	21.7	Not as good as 3% PVP; many nuclei burst; granularity of nuclei fades rather soon.
PVP + Dextrose	2.2 2.2	-	-	Granular structure of nuclei poor.
Raffinose	10	-	-	Nuclei damaged; no nucleoli seen; broken nuclei abundant; swollen and crinkled nuclei seen.
Raffinose	5	-	-	Same as 10% raffinose.
Sucrose	8.5	10	21.3	Nuclei "washed out" or agranular as in dextrose; nucleoli fade at once; many nuclei burst after 5 to 10 min; nuclei not plentiful, suggesting loss during homogenization.
Tween 80	1	-	-	Many nuclei and fine particulates; nucleoli quite clear and numerous; central area of nuclei somewhat agranular.

TABLE 33 (Contd.)

Medium	Concentration, %	Number of nuclei measured	Average nuclear diameter, μ	Comments
Tween 80	3	-	-	Nuclei better than those in 1% or 5% Tween 80; nucleoli more distinct, nuclei more granular in the center; many fine cytoplasmic particles.
Tween 80	5	-	-	Nuclei intact, but nucleoli not very distinct; nuclei highly granular.
Ringer's solution		-	-	(NaCl 8.6 g; CaCl ₂ 0.33 g; KCl 0.30 g; + glass-distilled water to make 1 liter.) Nuclei fair, granular, but nucleoli indistinct; nuclei small to medium in size, not swollen; many fine cytoplasmic particulates appeared in or on tiny vesicles, floating in homogenate medium.
NaCl	0.5	-	-	Nuclei essentially without nucleoli, somewhat small, less uniform in size, less granular.
Kassel-Kopac salt medium ⁽⁸⁾		20	21	Nuclei highly granulated but abnormal, containing a central contracted area surrounded by a space with nucleoli "attached" normally to the inner surface of the nuclear envelope; many nucleoli broken up.
Chalkley's salt medium ⁽⁹⁾		-	-	Nucleoli faded nearly as fast as in dextrose or sucrose; this medium poor for nuclei.

*Polyvinyl pyrrolidone

Electron microscopy of homogenates. Homogenates of *P. carolinensis* made of 8.5% sucrose and centrifuged in sucrose gradient tubes had well-maintained vesicles (Figure 29) and mitochondria which were very similar in detail to those in sectioned whole amoebae (Figure 30). Mitochondria showed no significant swelling, and the tubules appeared to be essentially normal. PVP preserved the fine structure of the mitochondria quite well (Figure 31). However, dextrose and PVP together (Figure 32) were not significantly better than 3% PVP alone, and neither of these was as good as sucrose for preserving the normal morphology. Nuclei were best maintained in PVP although even this medium damaged them considerably.

Figure 29. *Pelomyxa carolinensis*: Vesicles (V) from Fraction No. 1 of a sucrose gradient tube.

Figure 30. *Pelomyxa carolinensis*: Mitochondria (M) with some vesicles from the mitochondrial fraction of a sucrose gradient tube (Fraction No. 2)

Figure 31. Pelomyxa carolinensis: Mitochondria from a homogenate made in 3% PVP

Figure 32. Pelomyxa carolinensis: Mitochondria from a homogenate made in 3% PVP with 4.5% dextrose

The mitochondria of P. illinoisensis shown in Figure 33 were prepared in PVP and dextrose in concentrations near those used for P. carolinensis (Figure 32). The mitochondria of P. illinoisensis were more severely damaged, showing pronounced swelling and separation of the outer membrane. There was little change in the fine structure of various small vesicles when P. illinoisensis was homogenized in 2.5% Ficoll (Figure 34).

Figure 33. *Pelomyxa illinoisensis*: Mitochondria and vesicles from a homogenate made in 2.2% PVP with 4.5% dextrose. Note separation of outer mitochondrial membrane.

Figure 34. *Pelomyxa illinoisensis*: Vesicles of various types from the light fraction of a centrifuged homogenate made in 2.5% Ficoll. V, vesicles; PV, pinocytosis vesicles; FV, flattened vesicles.

The nuclei were damaged somewhat more than the vesicles, although they remained whole and an occasional nucleolus could be seen. Mitochondria also were damaged although the damage was somewhat less than that seen in Figure 33. The mitochondria in a control amoeba are shown in Figure 35. The tubules of normal mitochondria in this species, unlike those in P. carolinensis, are almost entirely limited to the peripheral region.

Figure 35. Pelomyxa illinoisensis: Control section through an amoeba showing mitochondria and cytoplasm (CY). Ribosomes and vesicles can be seen in the cytoplasm, suspended in a groundplasm.

It appeared from these results that a fractionation medium that was suitable for one species might not be as suitable for another, and that a medium in which morphology of nuclei was well maintained might not preserve the ultrastructure of other subcellular particles.

Injection of *Pelomyxa illinoisensis* homogenates into supralethally X-irradiated *Pelomyxa illinoisensis*

The least toxic of the media (Table 32) were used in optimum concentrations for the subcellular components of interest. Ficoll (4%) and glycerol (60%), which preserved the nuclear structures exceptionally well according to phase microscopy, were used as the homogenizing media. Homogenates were made in 4% Ficoll and injected into 15 supralethally irradiated amoebae, but none survived longer than the 200 irradiated, untreated controls. Another homogenate was made using 60% glycerol. Some of this was injected into 20 supralethally X-irradiated amoebae, but

all of these died within a week as did 100 irradiated, untreated controls. The 60% glycerol homogenate was also used to bathe 75 irradiated amoebae, but these also died within a week.

P. illinoisensis amoebae were then homogenized in 2.2% PVP to which had been added 2.2% dextrose. A portion of this homogenate was concentrated and injected into 34 irradiated P. illinoisensis. Again, none of these lived longer than the 200 irradiated controls.

In previous work,⁽⁵⁾ injection of 8.5% sucrose homogenate into more than 200 lethally irradiated amoebae did not prolong survival time. Similarly, no restoration was obtained in 55 irradiated amoebae injected with 0.25 M dextrose homogenate of P. illinoisensis.

Conclusions

On this and a previous occasion⁽⁵⁾ attempts have been made to prevent death in P. illinoisensis amoebae by injecting various types of homogenates from unirradiated donor P. illinoisensis organisms. None of these preparations has prevented death. However, recovery can be obtained after direct transfer of unirradiated protoplasm from living cells⁽¹⁾ or by certain strata from centrifuged living cells⁽²⁾ or by centrifuged, enucleated strata of living cells.⁽¹⁰⁾ When the amoebae are broken by homogenization, the activity required for radio restoration is lost. None of our attempts to vary the homogenization procedure in order to retain the radio restorative activity has been successful. Some other possibilities are the lowering of the temperature of the homogenates to 0°C or below, and reducing the time from homogenization to injection (this time has varied from 15 to 45 min or more).

Although radio restorative activity is lost in our homogenates, certain of the media maintain the fine structure of many protoplasmic components in a near normal state. These media will be useful in future biochemical studies on these amoebae.

References

1. Daniels, E. W. X-irradiation of the giant amoeba, Pelomyxa illinoisensis. I. Survival and cell division following exposure. Therapeutic effects of whole protoplasm. J. Exptl. Zool. 130, 183-197 (1955).
2. Daniels, E. W. Recovery from lethal X-radiation injury in amoebae after injection of centrifugal portions of nonirradiated cells. Radiation Res. 5, 604-605 (1956).

3. Daniels, E. W. X-irradiation of the giant amoeba, Pelomyxa illinoi-sensis. II. Further studies on recovery following supralethal expo-sure. J. Exptl. Zool. 137, 425-442 (1958).
4. Daniels, E. W., and L. E. Roth. X-irradiation of the giant amoeba, Pelomyxa illinoisensis. III. Electron microscopy of centrifuged organ-isms. Radiation Res. 14, 66-82 (1961).
5. Daniels, E. W. Microinjection studies on amoebae after their expo-sure to a supralethal dose of X-rays or γ -rays. Biological and Medical Research Division Semiannual Report, January through June 1959, Argonne National Laboratory. ANL-6200, pp. 82-88.
6. Thomson, J. F. Isolation of cytoplasmic particulates from animal tissues by density-gradient centrifugation. Anal. Chem. 31, 836-838 (1959).
7. Palade, G. E. A study of fixation for electron microscopy. J. Exptl. Med. 95, 285-298 (1952).
8. Kassel, R., and M. J. Kopac. Experimental approaches to the evalua-tion of fractionation media. I. The action of ions on the protoplasm of Amoeba proteus and Pelomyxa carolinensis. J. Exptl. Zool. 124, 279-302 (1953).
9. Kudo, R. R. Protozoology. Fourth Ed. Charles C. Thomas, Publ., Springfield, Illinois, 1954, p. 882.
10. Daniels, E. W. Recovery of reproductive function in supralethally X-irradiated amoebae following cytoplasmic microtransfer. Biologi-cal and Medical Research Division Semiannual Report, July through December 1961, Argonne National Laboratory. ANL-6535, p. 54.

PROGRESS REPORT: THE BIOSYNTHESIS OF METHIONINE.
UTILIZATION OF S-ADENOSYLMETHIONINE FOR
THE BIOSYNTHESIS OF METHIONINE

Stanley K. Shapiro, Phoebe Lohmar* and Mary Hertenstein**

It has now been established that S-adenosylmethionine is the methyl donor in a wide variety of transmethylation reactions.⁽¹⁾ In previous reports we have shown that this sulfonium compound may serve as a methyl donor in the methylation of homocysteine to methionine with cell-free extracts of bacteria, yeasts, rat liver, and plant seeds.^(2,3) However, except for its role as a methyl donor for the latter enzyme - S-adenosylmethionine: homocysteine methyl transferase - the sulfonium compound has not been shown to be a source of any other part of the methionine molecule in vivo or in vitro. In a previous report⁽⁴⁾ the utilization of S-adenosylmethionine for growth by various microorganisms has been described. In order to gain further insight into the function of S-adenosylmethionine, cultures that utilized the sulfonium compound for growth were grown in the presence of the compound labeled in various positions. This report will summarize the results and metabolic implications of these experiments.

Materials and Methods

Cultures The cultures used were derived from Aerobacter aerogenes ATCC 8724 and have been described previously.⁽⁴⁾ Culture 62 is a methionine mutant that does not utilize S-adenosylmethionine. Culture 68 is a mutant that utilizes either methionine or S-adenosylmethionine for growth while culture AM-1 responds to S-adenosylmethionine but not to methionine.

Compounds. All compounds used were commercial products except for S-adenosylmethionine. The latter was isolated from Saccharomyces cerevisiae according to the method of Schlenk et al.⁽⁵⁾ In order to obtain the sulfonium compound labeled with C¹⁴ in the methyl group, with S³⁵, and with C¹⁴ in carbon 2 of the methionine moiety of S-adenosylmethionine, the yeast cells were supplemented with L-methionine-C¹⁴H₃, -S³⁵ and -2C¹⁴, respectively. In each case a supplement of 2 μ moles methionine per ml of medium was used. Although the total yield of sulfonium compound is not as high as possible, this concentration results in more efficient utilization of labeled methionine, while still giving good yields of S-adenosylmethionine.

Growth of cells. The cultures were grown in the minimal medium described previously.⁽⁴⁾ Radioactive S-adenosylmethionine was used at a concentration of 0.2 μ moles per ml, which permits optimum growth of the

---

*Argonne Semester Student from Knox College.

**Argonne Semester Student from Grinnell College.

culture. The salts and glucose were sterilized separately, while the sulfonium compound was sterilized by filtration through Millipore filters. Ten ml of supplemented medium was inoculated from a 24-hr nutrient agar culture. After growth for 24 hr at 35°C the culture was added to 190 ml of medium. The cultures were incubated for 16 hr at 35°C on a Brunswick Gyrorotary shaker. All final cultures were checked for reversion as follows: one ml of the cells used for the fractionation procedure was washed twice in 0.1 M phosphate buffer pH 6.8, and 0.05-ml aliquots were added to 10 ml of minimal medium and to 10 ml of minimal medium plus S-adenosylmethionine and incubated for 24 hr at 35°C on a shaker. No growth was observed in minimal medium in any of the cases.

Fractionation of cells. The cells were collected by centrifugation at $8000 \times g$ for 30 min in a Lourdes centrifuge. The supernatant solution was saved and the cells were resuspended in 100 ml distilled H₂O and centrifuged as before. The wash water was also saved. The washed cells were then fractionated according to the procedure of Roberts et al.⁽⁶⁾ Minor modifications in this procedure will be indicated in the experimental section.

Isolation of methionine. After the fractionation procedure the resulting protein fraction was hydrolyzed with 1 ml of 6 N HCl per 50 mg wet weight of original cells. The hydrolysis was carried out in sealed ampoules at 110°C for two hr. This time of hydrolysis gave the highest yield of methionine. In order to remove the HCl the hydrolysate was evaporated to dryness in a rotary evaporator under reduced pressure, resuspended in 25 ml distilled H₂O four times and evaporated to dryness each time. The dried hydrolysate was reconstituted with 1 ml H₂O per g wet cells. In order to minimize oxidation of methionine during chromatography, 0.1 ml of a 1:100 dilution of thioglycol was added per gram of original cells.

Methionine was isolated from the protein hydrolysate by paper chromatography. Two hundred μ l of the hydrolysate was spread over a 30-cm band on Whatman No. 1 paper and 5 μ l of the hydrolysate was placed on each end of the paper as a reference spot. The chromatograms were developed in a solution of n-butanol, water and glacial acetic acid (12:5:3). Platinic iodide was used to locate methionine ($R_f = 0.5$) in the reference strips at each end of the paper. The corresponding methionine area in the main part of the chromatogram was cut out in a 4-cm band. The material on the band was eluted with distilled H₂O. Usually five such eluates were combined and lyophilized. The dry residue was reconstituted with 1.0 ml distilled H₂O. To this was added 0.05 ml of 30% H₂O₂ in order to oxidize methionine to its sulfoxide. Methionine sulfoxide was isolated from this solution by preparing bands and reference spots on paper chromatograms exactly as before. After development of the papers in the solvent system used before, the methionine sulfoxide was located in the reference strips ($R_f = 0.2$) with ninhydrin. The methionine sulfoxide was eluted from 3-cm bands, lyophilized, and redissolved in 1.0 ml distilled H₂O. The purity of

the final product was checked by co-chromatography with commercial samples of methionine sulfoxide in a number of solvent systems:

1. n-butanol, water, glacial acetic acid (12:5:3).
2. tert-butanol, 2-butanone, water, diethylamine (10:10:5:1).
3. n-butanol, water, glacial acetic acid (64:35:1).
4. ethanol, ammonia, water (18:1:1).
5. tert-butanol, formic acid, water (70:15:15).

In order to remove ammonium ions, all paper eluates were treated with Dowex 50. The concentration of methionine sulfoxide in these solutions was determined by the ninhydrin method, using a standard curve prepared with methionine sulfoxide.

Isolation of threonine. The location of threonine on the first paper chromatograms of the protein hydrolysates was determined by guide strips prepared with commercial DL-threonine. Bands of 4 cm were cut, eluted, and concentrated as described above for methionine. The concentrate from 5 bands was reconstituted in one ml distilled water. Threonine was isolated from the other amino acids in this solution by chromatography in solvent 2. After location of the threonine band with the aid of guide strips, the amino acid was eluted and concentrated. The purity of the final product was checked by co-chromatography with commercial DL-threonine in the five solvents listed above. After pretreatment of the reconstituted final eluates with Dowex 50 the concentration of threonine was determined by the ninhydrin method with a standard curve prepared with threonine.

Radioactivity measurement. The location of radioactivity on paper chromatograms was determined with an automatic scanning and recording device.⁽⁷⁾ Radioactivity in various solutions was measured with a Tricarb Liquid Scintillation Spectrometer. All samples were made up to a total volume of 1.0 ml with distilled water. To this was added 20 ml of scintillation fluid consisting of 0.4% 2,5-diphenyloxazole in equal volumes of toluene and ethanol.

Results

Distribution of radioactivity. Since culture 62 does not grow on S-adenosylmethionine, methionine was added to this culture in order to obtain growth in the presence of the labeled sulfonium compound. With the three labels used, there was no uptake of radioactivity by the cells, since virtually all the radioactivity could be accounted for in the supernatant fluid. These cells were not treated any further. Cultures 68 and AM-1 showed good uptake of radioactivity. The distribution of radioactivity among the various fractions when these two cultures were grown on C¹⁴H₃-labeled

S-adenosylmethionine is shown in Table 34. It may be seen that the alcohol-, ether-soluble fraction (lipids and phospholipids) of culture 68 contained 27% of the label incorporated, whereas this fraction of culture AM-1 contained 34% of the label incorporated by the cells. The radioactivity remaining in the alcohol-soluble, ether-insoluble fraction was attributed to alcohol-soluble proteins and contained 8% and 11% of the activity incorporated by the cells of cultures 68 and AM-1, respectively.

TABLE 34

Distribution of radioactivity in cellular fractions of cultures 68 and AM-1 grown on S-adenosylmethionine- $C^{14}H_3$

Fraction	Total counts/min ($\times 10^3$)		% of total counts added		% of total counts incorporated	
	68	AM-1	68	AM-1	68	AM-1
Supernatant	1,785	3,217	31.3	56.4	-	-
Cold TCA	131	140	2.3	2.5	3.3	5.6
Alcohol-soluble	325	260	5.7	4.6	8.3	10.5
Alcohol-, ether-soluble	1,040	850	18.2	14.9	26.6	34.2
Hot TCA	132	98	2.3	1.7	3.4	3.9
Protein	2,140	1,270	37.5	22.3	54.7	51.1

Over 50% of the incorporated label in both cultures was accounted for by the protein fraction. Paper chromatograms of the protein hydrolysates were scanned for radioactivity and the only significant peak was observed at the R_f of methionine. After elution of bands, oxidation with H_2O_2 , and re-chromatography, the only radioactivity detected was at the R_f of methionine sulfoxide.

The distribution of radioactivity among the various fractions when the two cultures were grown on S^{35} -labeled S-adenosylmethionine is shown in Table 35. It may be seen that in this case the protein fractions of cultures 68 and AM-1 contained 77% of the radioactivity incorporated by the cells. In both cases approximately 10% of the activity was found in the alcohol-soluble protein fraction. Again, the only labeled amino acid detected was methionine. No radioactivity was observed at the R_f of cysteine on chromatograms made from the protein hydrolysates of either culture.

TABLE 35

Distribution of radioactivity in cellular fractions of cultures 68 and AM-1 grown on S-adenosylmethionine-S³⁵

Fraction	Total counts/min ($\times 10^3$)		% of total counts added		% of total counts incorporated	
	68	AM-1	68	AM-1	68	AM-1
Supernatant	1,600	3,618	25.4	57.4	-	-
Cold TCA	101	152	1.6	2.4	2.1	5.7
Alcohol-soluble	536	334	8.5	5.3	11.4	12.5
Alcohol-, ether-soluble	16	4	0.3	0.1	0.3	0.1
Hot TCA	207	123	3.3	1.9	4.4	4.6
Protein	3,600	2,080	57.1	33.1	76.6	77.6

Table 36 shows the distribution of radioactivity in fractions from cultures grown on S-adenosylmethionine labeled with C¹⁴ in carbon 2 of the methionine moiety of S-adenosylmethionine. Here again the only fraction with large amounts of radioactivity was the protein fraction. This fraction of cultures 68 and AM-1 contained 78% and 68%, respectively, of the radioactivity incorporated by the cells. As in the previous case, about 10% of the activity was in the alcohol-soluble protein fraction. Scanning chromatograms of protein hydrolysates of both cultures revealed radioactivity in several areas, including the methionine and threonine areas.

TABLE 36

Distribution of radioactivity in cellular fractions of cultures 68 and AM-1 grown on S-adenosylmethionine-methionine 2C¹⁴

Fraction	Total count/min ($\times 10^3$)		% of total counts added		% of total counts incorporated	
	68	AM-1	68	AM-1	68	AM-1
Supernatant	1,938	2,660	38.1	52.4	-	-
Cold TCA	163	153	3.2	3.1	5.2	6.3
Alcohol-soluble	269	320	5.3	6.3	8.6	13.2
Alcohol-, ether-soluble	15	6	0.3	0.1	0.5	0.3
Hot TCA	254	179	5.1	3.5	8.1	7.4
Protein	2,440	1,650	48.1	32.5	77.7	68.2

Isolation of methionine. The procedure described yielded pure methionine sulfoxide as determined by paper chromatography of the final product in five solvent systems. Commercial L-methionine sulfoxide was used to establish a standard curve for the ninhydrin determination. Methionine sulfoxide was added to the Dowex-treated paper eluates in order to demonstrate the quantitative recovery of the added compound under these conditions.

The specific activity of the methionine sulfoxide was compared with that of the S-adenosylmethionine used in each type of experiment. The results obtained from a typical series of experiments are summarized in Table 37. It may be seen that 82% and 78% of the protein methionine is radioactive when culture 68 is grown on S-adenosylmethionine labeled with S^{35} or $C^{14}H_3$, respectively. Under the same conditions 50% and 59% of the protein methionine of culture AM-1 contains S and CH_3 , respectively, which originated in S-adenosylmethionine. The data also show that there is virtually no incorporation of label into methionine when either culture is grown on S-adenosylmethionine labeled with C^{14} in carbon 2 of the methionine moiety.

TABLE 37

Utilization of S-adenosylmethionine (AM) for
the biosynthesis of protein methionine*

Label in AM**	Per cent incorporation of label into methionine†	
	Culture 68	Culture AM-1
Methyl C^{14}	77.8	58.8
S^{35}	82.3	49.6
Methionine $2C^{14}$	1.5	3.7

*Methionine isolated as methionine sulfoxide.

**Specific activity of AM in counts/min/ μ mole: Methyl C^{14} , 135,000; S^{35} , 152,000; Methionine $2C^{14}$, 145,000.

†Specific activity of methionine sulfoxide (x 100) divided by specific activity of AM.

Isolation of threonine. Since the results described above suggested that only the S and CH_3 groups of S-adenosylmethionine are utilized for methionine synthesis, it seemed worth while to determine the fate of the four-carbon chain of the sulfonium compound. In the experiments with $2C^{14}$ -labeled S-adenosylmethionine there was considerable radioactivity at the R_f of threonine in paper chromatograms of the protein hydrolysates. Therefore, threonine was isolated as described above. The isolated threonine from cultures AM-1 and 68 showed 14% and 8%, respectively, of the specific activity of the sulfonium compound.

Discussion

In a previous report from this Laboratory⁽⁴⁾ it has been emphasized that S-adenosylmethionine does not support the growth of most methionine mutants. The results of the experiments described here demonstrate that S-adenosylmethionine is not a source of the intact methionine molecule in the two cultures that utilize the sulfonium compound for growth. Both cultures utilize only the S and CH₃ groups of the sulfonium compound for the biosynthesis of methionine. Culture 68 is a mutant that grows when supplied with methionine or S-adenosylmethionine.⁽⁴⁾ The fact that most of the resultant protein methionine of culture 68 contains S³⁵ and C¹⁴H₃ suggests that under these conditions this mutant depends entirely on S-adenosylmethionine for the biosynthesis of methionine. On the other hand, culture AM-1 does not grow on methionine⁽⁴⁾ and the results obtained with labeled S-adenosylmethionine show that the culture is able to synthesize more than half of its methionine from unlabeled compounds. This supports the concept that the genetic block in this culture has little to do with the pathway of methionine biosynthesis. Presumably the same enzymatic decomposition products of S-adenosylmethionine that are required for the growth of culture 68 may also be utilized by culture AM-1, resulting in radioactive protein methionine. The mechanism whereby this occurs is unclear. Although the results with both cultures are compatible with the idea of a "transmethylation" the present experiments do not prove that the S and CH₃ are transferred as a unit.

Both cultures that utilize the sulfonium compound for growth have potent S-adenosylmethionine:homocysteine methyl transferases,^(2,4) promoting the reaction:

However, if this reaction alone accounted for the growth of the organisms, one would not expect the incorporation of the S atom into methionine. This could be explained if the demethylated sulfonium compound - S-adenosylhomocysteine - were decomposed enzymatically to homocysteine. However, in this case the carbon chain of the resultant methionine should be radioactive, and the results show that it is not.

In addition to the methyl transferase these organisms also contain enzymes which decompose S-adenosylmethionine as follows:⁽⁸⁾

It is possible that 5'-methylthioadenosine, methylthioribose, or further decomposition products enter the pathway of methionine biosynthesis in some unknown manner. Since culture 68 is presumably blocked somewhere before this point, it utilizes S-adenosylmethionine as a source of 80% of the S and CH₃ groups of methionine. Culture AM-1 is not a methionine mutant and therefore should be able to synthesize its protein methionine. This is corroborated by the finding that the incorporation of the S and CH₃ groups into the protein methionine of culture AM-1 is not complete. This could result from incorporation of the enzymatic decomposition products of S-adenosylmethionine via the hormonal pathway. This mutant requires S-adenosylmethionine itself or some as yet unidentified derivative of this compound for growth. Some support for the latter concept is given by the isolation of radioactive threonine from both cultures when grown on S-adenosylmethionine-2C¹⁴. Homoserine is derived non-enzymatically from the enzymatic decomposition product α -amino, γ -butyrolactone. Consequently, one would expect the radioactive homoserine to result in radioactive threonine.

The experiments with C¹⁴H₃-labeled S-adenosylmethionine revealed extensive incorporation of the label into the lipid and phospholipid fraction of the cells (Table 34). This role of S-adenosylmethionine has been surmised from earlier experiments with C¹⁴H₃-methionine carried out with various methionine-requiring bacteria. In recent experiments with the mutants described in this report and C¹⁴H₃-labeled S-adenosylmethionine, O'Leary has shown that the sulfonium compound is utilized in the biosynthesis of bacterial fatty acids.⁽⁹⁾

Summary

The role of S-adenosylmethionine in the biosynthesis of methionine has been investigated with two mutants of Aerobacter aerogenes that utilize the sulfonium compound for growth. The results show that only the S and CH₃ groups of S-adenosylmethionine are utilized for the synthesis of methionine. Carbon atom 2 of the methionine moiety of the sulfonium compound is not utilized. When S-adenosylmethionine labeled with C¹⁴ in the methyl group was used, over one-fourth of the radioactivity incorporated by both cultures was associated with the lipid fraction of the cells.

References

1. Shapiro, S. K., and F. Schlenk. The biochemistry of sulfonium compounds. Advances in Enzymol. 22, 237-280 (1960).
2. Shapiro, S. K., and D. A. Yphantis. Assay of S-methylmethionine and S-adenosylmethionine homocysteine transmethylases. Biochim. Biophys. Acta 36, 241-244 (1959).

3. Turner, J. E., and S. K. Shapiro. S-methylmethionine and S-adenosylmethionine-homocysteine transmethylese in higher plant seeds. *Biochim. Biophys. Acta* 51, 581-584 (1961).
4. Shapiro, S. K. Utilization of S-adenosylmethionine by microorganisms. *J. Bacteriol.* 83, 169-174 (1962).
5. Schlenk, F., J. L. Dainko, and S. M. Stanford. Improved procedure for the isolation of S-adenosylmethionine and S-adenosylethionine. *Arch. Biochem. Biophys.* 83, 28-34 (1959).
6. Roberts, R. B., P. H. Abelson, D. B. Cowie, E. T. Bolton, and R. J. Britton. *Studies of Biosynthesis in Escherichia coli*. Carnegie Institution of Washington Publication 607, Washington, D. C. (1955).
7. Eisler, W., W. Chorney, and W. E. Kisielleski. An automatic scanner for tritiated compounds in paper chromatograms. *Abstr. Am. Chem. Soc. Meeting, San Francisco*, P 12 C (1958).
8. Shapiro, S. K., and A. N. Mather. The enzymatic decomposition of S-adenosyl-L-methionine. *J. Biol. Chem.* 233, 631-633 (1958).
9. O'Leary, W. M. S-adenosylmethionine in the biosynthesis of bacterial fatty acids. *J. Bacteriol.* 84, 967-972 (1962).

PROGRESS REPORT: BIOSYNTHESIS OF METHIONINE FROM
HOMOCYSTEINE AND SULFONIUM DERIVATIVES
IN ESCHERICHIA COLI. II.

Morris Pfeffer

The microorganisms in use in this study now include Escherichia coli 113-3 and K-12, as well as E. coli (Texas) and (Texas) M. This report deals primarily with results obtained with the sulfonium compound S-adenosylmethionine (S-AM).

Cell-free extracts of all four strains of E. coli were examined for methionine biosynthetic activity by the method of Shapiro and Yphantis⁽¹⁾ using the labeled substrates S-AM-C¹⁴H₃, S-AM-S³⁵, and S-AM-2C¹⁴. It was found, and verified chromatographically (see Reference 2 for methods), that equal amounts of labeled methionine are formed from each of the three substrates (see Table 38). If the methionine were being formed by the process of transmethylation, only the methionine synthesized from S-AM-C¹⁴H₃ would be labeled. The reaction occurred only in the presence of homocysteine. Thus it was not simple splitting but involved the following mechanism:

The enzyme mediating the reaction has been tentatively named S-AM homocysteine adenosyltransferase. Confirmatory investigations are under way. Methionine synthesized from any of the three labeled forms of S-AM by E. coli (Texas) M had the same specific activity as the corresponding precursor (see Table 39). The other three strains of E. coli are being analyzed in the same way. In addition, homocysteine-S³⁵ and unlabeled S-AM will be used to determine whether, under these circumstances, S-adenosylhomocysteine (S-AH) is labeled and methionine unlabeled.

TABLE 38

Biosynthesis of methionine by cell-free extracts
of E. coli, m μ moles/ml/hr

Substrate	<u>E. coli</u> strain			
	(Texas)*	(Texas) M	K-12*	113-3**
S-AM-C ¹⁴ H ₃	25.4	21.7 [†]	19.8	98.0
S-AM-S ³⁵	24.0	27.6	22.2	95.8
S-AM-2C ¹⁴	25.0	16.0	21.2	96.2

*S-AM: 0.2 μ moles/ml; homocysteine: 8 μ moles/ml

**S-AM: 16 μ moles/ml; homocysteine: 16 μ moles/ml

[†]Methionine determined by measurement of radio-activity (Reference 1).

TABLE 39

Specific activities, cpm/ μ mole, of methionine synthesized in
cell-free extracts of E. coli (Texas) M

Substrate	Substrate specific activity	Methionine specific activity
S-AM-C ¹⁴ H ₃	159,000	127,000
S-AM-S ³⁵	24,000	29,250
S-AM-2C ¹⁴	600,000	580,000

Reaction mixture contained: S-AM, 0.2 μ mole/ml; homocysteine, 8 μ moles/ml; cell-free extract, 2 ml, 0.1 M phosphate buffer, pH 6.8; total volume, 10 ml. Incubated 1 hr at 37°C. Methionine specific activity determined according to method of Shapiro et al.(2)

A previous report(3) described the start of a series of experiments dealing with the utilization, by the auxotroph E. coli (Texas) M, of various labeled substrates capable of promoting the growth of this microorganism. Cultures were grown on suboptimal amounts (15 m μ moles/ml) of the substrates for 18 hr at 37°C on a rotary shaker. The cells were fractionated by the method of Roberts et al.,(4) the protein was hydrolyzed and the concentration and specific activity of the methionine were determined. The results of these experiments are presented in Table 40. At the time of

the last progress report, the only experiments which had been performed were those with S-methylmethionine (S-MM), and the lack of the S-MM methyl label in the protein methionine was unexplained. However, as the experiments were continued, several points became clear. Methionine, S-MM and S-AM are not incorporated as methionine, are not broken down to yield methionine, and do not produce methionine by some simple group transfer. The marked increase noted in protein methionine makes it appear that these compounds are serving as the source of an intermediate that is catalytically active in methionine biosynthesis.

TABLE 40

Biosynthesis of methionine by 18-hr cultures of E. coli (Texas)
and E. coli (Texas) M

Substrate	Strain	Total uptake, μ moles	Protein methionine, μ moles	Increase in methionine, %	Incorporation of label into protein methionine, %
L-Methionine- $C^{14}H_3$	Wild type	2.40	50.4	2100	0.11
	Mutant	2.36	34.5	1400	0.19
L-Methionine- S^{35}	Wild type	2.84	62.2	2200	0.49
	Mutant	2.40	22.6	900	3.8
S-MM- S^{35}	Wild type	1.50	55.2	3700	1.3
	Mutant	1.47	62.2	4200	0.7
S-MM- $2C^{14}$	Mutant	18.6	197.3	1100	2.4
S-AM- $C^{14}H_3$	Mutant	1.60	47.3	3000	<0.02
S-AM- S^{35}	Wild type	1.40	51.1	3700	<0.004
	Mutant	1.36	38.7	2800	0.02

To test this hypothesis, resting cell suspensions of E. coli (Texas) M were added to a medium containing glucose and DL-methionine- $2C^{14}$, harvested at 20 and 40 min and extracted with cold 1.5 N perchloric acid (PCA). S-AH was recovered from the PCA extract and was found to have 90% of the methionine specific activity at 20 min and 86% at 40 min. This is interpreted to mean that the methionine taken up by the cells is converted to S-AM. S-AM is active not only in methionine biosynthesis but in several other vital reactions. In these other reactions it acts as a source of methyl groups, yielding labeled S-AH after the reactions have taken place. Only the methionine biosynthetic reaction uses up the total methionine moiety of S-AM, and this is the reaction responsible for the slow diminution in S-AH specific activity. This mechanism is predicated on the assumption that S-AH can be regenerated to S-AM by the uptake of one-carbon units from the folate series of coenzymes. We plan to test this assumption in future experiments.

References

1. Shapiro, S. K., and D. A. Yphantis. Assay of S-methylmethionine and S-adenosylmethionine transmethylases. *Biochim. Biophys. Acta* 36, 241-244 (1959).
2. Shapiro, S. K., P. Lohmar, and M. Hertenstein. Utilization of S-adenosylmethionine for the biosynthesis of methionine. *Arch. Biochem. Biophys.* 100, 69-74 (1963).
3. Pfeffer, M., and S. K. Shapiro. Progress report: Biosynthesis of methionine from homocysteine and sulfonium derivatives in Escherichia coli. Biological and Medical Research Division Semiannual Report, January through June 1962, Argonne National Laboratory. ANL-6723, pp. 165-173.
4. Roberts, R. B., P. H. Abelson, D. B. Cowie, E. T. Bolton, and R. J. Britten. Studies of biosynthesis in Escherichia coli. Carnegie Inst. Pub. 607, Washington, D. C., 13-30 (1955).

ULTRAVIOLET MICROSCOPY OF PURINE COMPOUNDS IN THE YEAST VACUOLE

George Svihla, Julia L. Dainko, and F. Schlenk

The prominence of the vacuole in the yeast cell is in striking contrast to the limited amount of study devoted to this structure, and the understanding of its function has remained rather scanty. Much has been inferred from observations on vacuoles in higher plants, and functions such as waste receptacle, storage of reserve material, and osmotic regulator have been suggested.⁽¹⁾

Localization of a cellular constituent, S-adenosylmethionine, in the vacuoles of Candida utilis and Saccharomyces cerevisiae has been observed by ultraviolet photomicrography.^(2,3) Therefore, it was tempting to search for other ultraviolet-absorbing compounds in the vacuole and to study their fate in cellular metabolism. Through such studies generalizations about the function of this cellular compartment may eventually become possible.

Purines, pyrimidines and their nucleosides appeared to be likely materials. The utilization of such compounds in the absence of other assimilable nitrogen sources has been studied by Di Carlo et al.,⁽⁴⁾ but no cytological data were included in this work. More recently, Roush et al.⁽⁵⁾ published an elaborate study of the uptake of purine bases by C. utilis. Roush⁽⁶⁾ observed crystals of various purines in the vacuole by conventional microscopy. This gave further encouragement to our cytological study.

Materials and Methods

C. utilis ATCC 9950 and S. cerevisiae ATCC 7752 were cultured as specified earlier.^(7,8) The washed cells were stored at 4°C until use. For experiments with the purines, pyrimidines, and other ultraviolet-absorbing compounds the same salt medium was used without ammonium sulfate. Glucose in a concentration of 3.0% was provided to sustain metabolism for a prolonged period.⁽⁵⁾ Most of the purine and pyrimidine compounds were present in a concentration of 1.0 or 2.0 μ moles/ml. The concentration of yeast at the outset of the experiment was 5 mg of moist cells per ml. Cultures were agitated on a rotary shaker at 30°C, and samples were withdrawn at the times specified. After centrifugation, the supernatant fluid was used for ultraviolet spectrophotometry of the compound under investigation. If a decline in optical density indicated assimilation of the substance, ultraviolet photomicrography of the cells was carried out to locate the material if possible. In some experiments, compounds were used in quantities exceeding their solubility; the insoluble sediment in these instances disappeared gradually as assimilation by the yeast proceeded. Ultraviolet photomicrography was carried out as in previous studies.^(2,3,7,8)

Results

The choice of *C. utilis* in preference to *S. cerevisiae* for the present survey was motivated mainly by its superiority for ultraviolet photomicrography. Its cytoplasm does not contain numerous granules which interfere with observation of the vacuole, and it does not form spores. It soon became apparent that only a few of the purines were useful for our experiments. Because of the low solubility of many of the substances, it was

Figure 36. Assimilation of uric acid by yeast. Spectrophotometry at 290 mμ of samples of the culture medium was used as a measure of uptake by the cells. Details are given in the text.

impossible to provide high concentrations of them in the medium, and the compounds had to be actively concentrated by the cells. We have confirmed the observation of Roush et al.(5) that uric acid is most readily assimilated from the medium. The process is illustrated for various experimental conditions in Figure 36. Washed cells of a 40-hr culture of *C. utilis* (experiment 1) showed a lag period of uric acid utilization; this was less pronounced with cells from a 5-hr culture (experiment 2). Curve 3 illustrates the abolition of the lag when cells incubated for 5 hr in experiment 1 were washed and again exposed to uric acid. An incubation of 19 hr before the second exposure to uric acid did not significantly alter

the uptake (experiment 4). Washed cells of a 24-hr culture of *S. cerevisiae* did not take up uric acid (experiment 5); the concentration in the medium remained unchanged even after 24 hr.

Concomitant ultraviolet photomicrographs were made of *C. utilis* in the above experiments. The choice of wavelengths at which the pictures were taken was dictated by the absorption spectra of the compounds examined. Uric acid has a convenient absorption maximum at 290 mμ. Adenosine derivatives, including S-adenosylmethionine, have an absorption maximum at 260 mμ, similar to that of nucleic acids. Figures 37 and 38 illustrate cells during and after the uric acid uptake shown in experiment 1, Figure 36. Crystals begin to appear in the vacuoles after 1 hr; their quantity reaches a maximum after 2 hr and declines after 3 hr; the uric acid is now used as a source of nitrogen, and budding is extensive. With cells induced to use uric acid (Figure 36, experiment 3) the ultraviolet photomicrographic record (not shown) was the same as that in Figure 37 and 38, except for an acceleration of the events: the vacuoles were filled with uric acid crystals after 15 min, and the material had virtually disappeared after 90 min. Thus, the concentration mechanism is more efficient in these cells

Figure 37 Ultraviolet photomicrographs of *C. utilis* from experiment 1, Figure 36, taken at 260 m μ (left side) and 290 m μ (right side) The times of sampling were (beginning at top) 30 min, 1 hr and 2 hr

Figure 36 Ultraviolet photomicrographs of *C. utilis* from experiment 1, Figure 36, taken at 260 m (left side) and 290 m (right side) The times of sampling were (beginning at top) 3, 4, and 5 hr

than the degradation mechanism. If a significant increase in the degradative enzymes had occurred by previous exposure to uric acid, the second addition of this compound would have been metabolized before it had a chance to accumulate in the vacuole.

When saturation of the culture medium of *C. utilis* with uric acid is maintained by an excess of solid material, the cells grow well after the initial induction period. The vacuoles remain filled with uric acid. However, little, if any, is seen in the vacuoles of buds as long as they remain attached to the mother cells. This is in contrast to our earlier observations that S-adenosylmethionine is transferred from the vacuole of the mother cell to that of the bud.⁽³⁾

From the quantity and size of the crystals of uric acid and other purines, as well as from chemical analyses,^(5,6) it can be concluded that the storage capacity of the vacuoles is considerable. Our earlier experiments have shown that the concentration of S-adenosylmethionine dissolved in the vacuolar fluid may be greater than 10%.⁽⁷⁾ Attempts were made to explore the vacuolar capacity by the following experiments. *C. utilis* was grown in glucose-ammonia medium containing sulfur amino acids in order to accumulate S-adenosylmethionine in the vacuoles. After washing, the cells were suspended in medium containing 1.0 μ mole of uric acid per ml, under conditions identical with those in experiment 1, Figure 36, and Figures 37 and 38. The photomicrographic record at selected times is given in Figures 39 and 40. The assimilation of uric acid was found to be slightly delayed, but the material persisted somewhat longer than in the earlier experiments. The distribution of uric acid crystals in the vacuole, which also contained S-adenosylmethionine, was more uniform than that of vacuoles containing uric acid only (Figures 37 and 38). It should be noted that S-adenosylmethionine is visible in the vacuole at 260 $m\mu$ (Figure 39), but not at 290 $m\mu$. Uric acid is visible at both wavelengths, because it is present in crystalline form.

Quantitative determinations of S-adenosylmethionine and of uric acid were carried out with cells of the type shown in Figures 37-40. They were harvested after two hours in the uric acid medium, washed, and extracted with an excess of water at 100°C for 15 min; the precipitate was removed by centrifugation, and uric acid was determined in the supernatant fluid by spectrophotometry at 290 $m\mu$. S-Adenosylmethionine was extracted from the cells with cold 1.5 N perchloric acid and assayed by chromatography and spectrophotometry.⁽⁹⁾ The data are listed in Table 41. This experiment demonstrates that uric acid can be concentrated in the vacuole against a considerable gradient even when S-adenosylmethionine is already present in the vacuole. Conversely, when uric acid and the sulfur amino acids are present in the culture medium at the same time and constitute the sole nitrogen sources (Table 42), uric acid accumulates in the vacuole first, but it does not prevent the subsequent entry of S-adenosylmethionine, which is formed gradually.⁽¹⁰⁾ For comparison, data obtained with an equivalent amount of ammonium salt as the principal nitrogen source are listed.

Figure 39 Ultraviolet photomicrographs of *C. utilis* enriched with S-adenosylmethionine. The washed cells were exposed to uric acid (1.0×10^{-4} mole/ml) for 30 min, 1, and 2 hr (beginning at top); the photographs on the left side were taken at $260 \text{ m}\mu$, on the right side, $290 \text{ m}\mu$. Experimental procedures were the same as described for experiment 1, Figures 36 and 37.

Figure 10 Ultraviolet photomicrographs of *C. utilis* enriched with S-adenosylmethionine. The washed cells were exposed to uric acid ($1.0 \mu\text{mole/ml}$) for 3, 4, and 5 hr (beginning at top), the photographs on the left side were taken at $260 m\mu$, on the right side, $290 m\mu$. Experimental procedures were the same as described for experiment 1, Figures 36 and 37.

TABLE 41

Determination of uric acid and of S-adenosylmethionine in vacuoles of *Candida utilis**

Type and treatment of cells	S-adenosylmethionine extracted, $\mu\text{moles/g}$	Uric acid extracted, $\mu\text{moles/g}$	Estimated quantity of material in the vacuole, mg/cm^3
1. Cells from glucose, salts, NH_4^+ medium	1.7	-	-
2. Same as (1), suspended in 1.0 mM uric acid, for			
60 min		55	92
90 min		91	153
120 min		81.5	137
3. Cells from culture with sulfur amino acid supplement	25.5	-	102
4. Same as (3), suspended in 1.0 mM uric acid, for			
90 min		55	187
120 min	23.6	93.5	252
150 min		76	222
5 hr	23.8	-	96

*Experimental details are found in the text. The volume of the vacuolar fluid was estimated to be 10% of the total volume. The estimated quantities of material in the vacuoles do not include compounds other than S-adenosylmethionine and uric acid. Because of the insolubility of the latter, it is not possible to give molar concentrations.

TABLE 42

The formation of S-adenosylmethionine in *Candida utilis* with uric acid or ammonium salt as a source of nitrogen

Nitrogen source and its concentration in the medium, $\mu\text{moles/ml}$	Yield of cells,* g 100 ml	S-adenosylmethionine in the cells, $\mu\text{moles/g}$
Uric acid (7.5)	2.9	2.0
NH_4^+ (30)	3.0	1.0
Uric acid (7.5) + L-methionine (5.0) + L-homocysteine (5.0) + S-methyl-L-methionine (5.0)	1.9	31.0
NH_4^+ (30) + L-methionine (5.0) + L-homocysteine (5.0) + S-methyl-L-methionine (5.0)	2.2	30.0

*After a culture period of 44 hr at 30°C under vigorous aeration, the moist packed cells were weighed and analyzed by the method of Schlenk and DePalma.(9)

Purines taken up readily and accumulated in the vacuole by C. utilis include adenine, 2,6-diaminopurine, guanine, isoguanine, hypoxanthine, and xanthine.⁽⁵⁾ The appearance of the cells was not changed when all of these purine compounds were added to the medium simultaneously. If they had gained entry independently, vacuoles filled solidly with crystals would have been expected. This was not observed, and some mutual interference of the purine bases must be assumed.

There was no significant assimilation of cytosine, thymine, orotic acid, or pseudouridine in concentrations of 1.0 $\mu\text{mole/ml}$ of medium. Spectrophotometry of samples of the culture medium showed no change of optical density for periods of 4 to 6 hr. Uracil was taken up slowly by C. utilis after a lag period of 2 hr, but no ultraviolet-absorbing material could be observed in the vacuole. Analogous experiments with purine and pyrimidine ribosides and deoxyribosides were also negative. On prolonged culture adenosine is an adequate source of nitrogen for the growth of C. utilis. To promote diffusion of substantial amounts into the cells, experiments with saturated medium, containing about 25 μmoles of adenosine per ml, were carried out. However, no ultraviolet-absorbing material was found in the vacuole even under these conditions. The cytoplasm showed relatively high density at 260 $m\mu$ in the early phases, and lobed or multiple vacuoles were seen in later stages. Further experiments were carried out with thiamine, riboflavin, nicotinic acid, nicotinamide, and the ultraviolet-absorbing amino acids L-tryptophan and L-tyrosine. None of these compounds was readily taken up from the medium or concentrated in the vacuoles of C. utilis.

Discussion

One of the serious limitations of ultraviolet photomicrography of the purine compounds studied here is their low solubility. The properties of uric acid may serve as an example. For simplicity, it is assumed that its solubility in the vacuolar fluid is similar to that in the culture medium (1.6 $\mu\text{moles/ml}$), and that the diameter of the average vacuole is 3 μ . From the molar extinction of uric acid, $E_m(290\text{ }m\mu) = 12,400$, it can be calculated that the optical density of a saturated solution, measured over a light path of 3 μ , would be only 0.006. Against the background of cytoplasmic constituents, this is not registered by the photographic film, and it is no surprise that the vacuole appears empty except for the optically dense uric acid crystals. Saturated isoguanine solution is about twice as absorbent, but even a saturated solution of adenine, measured at its absorption maximum at 261 $m\mu$ in a layer of 3 μ , would show an optical density of only 0.026. These calculations, based on physical constants compiled by Levene and Bass⁽¹¹⁾ and by Bendich⁽¹²⁾ emphasize the limitations of the free purines as optical tracers in yeast cytology. In the present study, the sole criterion for accumulation of the free purine compounds in the vacuole had to be the appearance of crystals, and valid statements about the transfer

of extraneous material can be made only for the extreme cases in which the limit of solubility is exceeded. There may be uptake and release at lower concentrations which are not observable by ultraviolet photomicrography. This limitation of the biological purines is in striking contrast to the usefulness of the highly soluble S-adenosylmethionine.⁽⁷⁾ Concentrations of this compound may range between 100 and 300 $\mu\text{moles/ml}$ of vacuolar fluid, if it is assumed that the vacuole comprises about 10% of the cellular space. With $E_m(260\text{ m}\mu) = 15,400$, the optical densities under these conditions range between 0.47 and 1.4 for a distance of 3μ .

The present study confirms earlier observations on the inability of S. cerevisiae to use the ring nitrogen atoms of the purine system.^(4,13) In this organism S-adenosylmethionine is still the only compound that has been found in the vacuole by ultraviolet microscopy. In contrast, C. utilis assimilates most of the biological purine compounds with ease, as shown by the extensive survey of Roush et al.⁽⁵⁾ Since a prerequisite for success with this technique is accumulation in the cell in high concentration, the non-assimilation of pyrimidines and purine and pyrimidine nucleosides restricts the number of useful compounds. Many compounds listed as negative in this survey are known to enter the cells, but the process is either too slow or the catabolism too fast to permit accumulation in the cells.

Because of the variation in uptake, distribution, and breakdown of the compounds that are convenient optical tracers, it is difficult to make generalizations about the vacuole on the basis of the present experiments. From the observation that several purines are so concentrated in the vacuole that crystallization results, it may be assumed that the vacuolar membrane possesses an active transport system. There is also a selective retention. Uric acid is an example of a compound that is readily introduced into, and removed from, the vacuole. Isoguanine, however, like S-adenosylmethionine, persists in the vacuole regardless of the metabolic conditions to which the cell is exposed; a lack of cellular enzymes for catabolism may be the cause. S-Adenosylmethionine readily finds its way into the vacuole of the bud, while the crystals of isoguanine and of uric acid remain in the vacuole of the mother cell; this may be explained by the limited solubilities of these compounds. Some properties of the vacuolar membrane and the cell membrane of C. utilis differ markedly. S-Adenosylmethionine penetrates the cell membrane slowly when supplied from the medium,⁽³⁾ but it gets into the vacuole readily when produced by endogenous synthesis. Conversely, adenosine enters the cells⁽¹⁴⁾ but is not concentrated in the vacuole. Osmotic phenomena do not necessarily govern the uptake of material, as shown by the limited interference of uric acid and S-adenosylmethionine in the vacuole.

The present data bear out some of the earlier conjectures of various authors⁽¹⁾ that the vacuole can be a storage place for some nutrients as well as a repository for inert or surplus material. It must be remembered, however, that neither function is restricted to the vacuole; deposition of material can be observed also in other regions of the yeast cell.

References

1. Bautz, E. Die Morphologie der hefeartigen Pilze. Die Hefen, ed. F. Reiff, R. Kautzmann, H. Luers, and M. Lindemann. H. Carl, Nuernberg, Vol. I, 1960. pp. 38-95.
2. Svihla, G., and F. Schlenk. Localization of S-adenosylmethionine in Candida utilis. J. Bacteriol. 78, 500-505 (1959).
3. Svihla, G., and F. Schlenk. S-Adenosylmethionine in the vacuole of Candida utilis. J. Bacteriol. 79, 841-848 (1960).
4. Di Carlo, F. J., A. S. Schultz, and D. K. McManus. The assimilation of nucleic acid derivatives and related compounds by yeasts. J. Biol. Chem. 189, 151-157 (1951).
5. Roush, A. H., L. M. Questiaux, and A. J. Domnas. The active transport and metabolism of purines in the yeast. Candida utilis. J. Cell. Comp. Physiol. 54, 275-286 (1959).
6. Roush, A. H. Crystallization of purines in the vacuole of Candida utilis. Nature (London) 190, 449 (1961).
7. Svihla, G., F. Schlenk, and J. L. Dainko. Some effects of ultraviolet irradiation on yeast cells (Candida utilis). Radiation Res. 13, 879-891 (1960).
8. Svihla, G., F. Schlenk, and J. L. Dainko. Spheroplasts of the yeast Candida utilis. J. Bacteriol. 82, 808-814 (1961).
9. Schlenk, F., and R. E. DePalma. The formation of S-adenosylmethionine in yeast. J. Biol. Chem. 229, 1037-1050 (1957).
10. Schlenk, F., J. L. Dainko, and S. M. Stanford. Improved method for the isolation of S-adenosylmethionine and S-adenosylethionine. Arch. Biochem. Biophys. 83, 28-34 (1959).
11. Levene, P. A., and L. W. Bass. Nucleic Acids. The Chemical Catalog Company, Inc., New York, 1931.
12. Bendich, A. Chemistry of purines and pyrimidines. The Nucleic Acids, ed. E. Chargaff and J. N. Davidson. Academic Press, New York, 1955. Vol. I, pp. 81-136.
13. Kerr, S. E., K. Seraidarian, and G. B. Brown. The utilization of purines and their ribose derivatives by yeast. J. Biol. Chem. 188, 207-216 (1951).
14. Duerre, J. A., and F. Schlenk. Formation and metabolism of S-adenosyl-L-homocysteine in yeast. Arch. Biochem. Biophys. 96, 575-579 (1962).

IONIC EQUILIBRIA OF THE NUCLEOSIDES

Lyle G. Bunville

In acid media protons are bound to deoxyribonucleic acid (DNA), the loci of these bound charges being the purine and pyrimidine residues of the double-helical polynucleotide chains comprising the DNA. In view of the role of these base residues in maintaining the helical (native) configuration of DNA through dispersive interaction with neighboring bases and through interchain hydrogen bonding to form the base pairs adenine-thymine and guanine-cytosine, it is of interest to have detailed information about the ionic equilibria of the free nucleosides.

The equilibrium of concern in the case of the protonated base is

with equilibrium constant $K_{\text{BH}} = (\text{B})(\text{H}_3\text{O}^+)/(\text{BH}^+)$, where (B) refers to the activity of the given species. Numerous results are given in the literature, but without specification of the ionic strength of the media, or temperature, and thus are not amenable to thermodynamic analysis. For this reason new determinations have been carried out at several ionic strengths using a potentiometric (glass electrode) method. The results for several of the nucleosides, expressed as $\text{pK}^\circ = -\log K^\circ$ for the ionization constant at zero ionic strength, 25.0°C, are: cytidine: $4.119 \pm .003$, deoxycytidine: $4.244 \pm .008$, and adenosine: $3.546 \pm .008$.

Additional work is in progress to extend the above list to the remaining nucleosides of interest and to determine the temperature coefficients of the various equilibrium constants.

PROGRESS REPORT: SOLUBLE PROTEINS FROM CELL NUCLEI

A. D. Barton

In a previous report,⁽¹⁾ we have described electrophoretic studies of the soluble proteins obtained by saline extraction of rat liver nuclei and other cell particulates isolated in sucrose media. Such studies are made difficult by the instability of some of the protein components. This difficulty was first encountered in earlier work,⁽²⁾ in which some of the protein precipitated in the descending limb when run in veronal buffer, and this was avoided by adding sodium chloride to the electrophoresis medium. However, even when the balanced medium of Sorof⁽³⁾ was used (sodium veronal, 0.02 M; NaCl, 0.03 M; pH 8.6) there was always some precipitation of protein during concentration and dialysis of the sample. This effect has been investigated in some detail, and as a result an improved method for isolating the nuclear proteins has been developed.

The effect of the precipitation of the proteins on the electrophoretic patterns is shown in Figure 41. All of the electrophoretic analyses in the present report were carried out in Sorof's balanced buffer, and all mobilities reported are in $(\text{cm}^2 \times 10^5)(\text{V})^{-1}(\text{sec})^{-1}$. Figure 41A gives the patterns of freshly prepared proteins obtained by saline extraction of nuclei isolated in 75 per cent sucrose without further washing.⁽⁴⁾ Figure 41B gives the

Figure 41. Moving boundary electrophoresis of soluble nuclear proteins. In all the figures, the ascending pattern is on the left and the descending on the right; the mobility scale at the bottom is in $(\text{cm}^2 \times 10^5)(\text{V})^{-1}(\text{sec})^{-1}$. A. Fresh preparation. B. The same preparation after prolonged dialysis. The dotted line shows the material lost during dialysis, as indicated by the differences between the two patterns.

patterns of the same preparation after additional dialysis for several days. This shows the effect in pronounced form, but the trend in this direction is perceptible after even two days.

The numbers in the descending patterns of Figure 41 give the relative amounts of protein corresponding to the different parts of the patterns, as indicated by the shift in the interference pattern, which is not shown in these tracings. The dotted pattern in Figure 41 shows the protein lost during prolonged dialysis, as indicated by the difference between the two patterns. There has been no loss of protein in the mobility range beyond -1.4, and only a small loss in the material with positive mobility. By far the greatest loss is in the mobility range +0.3 to -1.4. After removal of the unstable proteins, the material migrating beyond -1.5 now gives one clean peak, instead of being spread out. Except for the anomalous spike near +1 on the descending pattern, which is seen almost invariably, the material with positive mobility gives one clean peak near +0.7. The ascending and descending patterns now resemble one another very closely.

Careful tests indicated that there is no significant loss of protein through the dialysis membrane during concentration or dialysis. The half-time for escape of sucrose from the dialysis sac is two hours, and dialyzed preparations contain no anthrone-positive carbohydrate. The results of the color assay for protein agreed with quantities determined from the interference patterns obtained during electrophoresis, indicating the absence of any substantial amount of non-protein material.

In an effort to improve the preparation of the soluble nuclear proteins, the nuclei were isolated in 75 per cent sucrose and then washed in 30 per cent sucrose before extraction with saline. The proteins from the unwashed nuclei gave a pattern like that shown in Figure 42A; those from the washed nuclei gave one like that shown in Figure 42B. It is well known that isolation in aqueous media removes soluble protein from cell nuclei,⁽⁵⁾ and in this experiment the effect is increased by the wash in 30 per cent sucrose. Evidently, the more basic proteins (those with more positive mobility) resist this removal better than the less basic ones (those with more negative mobility).

The effect of maintaining better control of pH was tested by including phosphate buffer. The livers were perfused with a buffer solution, A, containing sodium chloride (0.12 M), and sodium phosphate (0.03 M), pH 7.3. They were homogenized in 75 per cent sucrose containing 10 per cent by volume of buffer A; the isolated nuclei were washed in 30 per cent sucrose, and were extracted with buffer A. A typical pattern is shown in Figure 43A. Evidently much of the material migrating in the range +1 to -1, including the component at +0.8, was removed during isolation of the nuclei; but the components at +0.3, -0.3, and -0.8 are prominently displayed. In addition, the phosphate extracted a large amount of protein migrating in

the range -3 to -4 which was not present in the previous extracts. It appeared that this was accompanied by damage to the nuclear membrane, since the nuclear pellets swelled enormously after the wash in 30 per cent sucrose.

Figure 42. Moving boundary electrophoresis of soluble nuclear proteins. A. Nuclei isolated in 75 per cent sucrose and extracted with isotonic saline. B. Same as in A, except that the isolated nuclei were washed with 30 per cent sucrose before extraction with saline.

When this procedure was repeated, adding CaCl_2 (0.18 mM) to the phosphate buffer A throughout, patterns like that shown in Figure 43B were obtained. The -0.3 and -0.8 components are still well displayed, although removal of the material in the range +1 to -1, and particularly the +0.8 component, is not so complete. The presence of the calcium prevented the extraction of the material in the range -3 to -4 and it prevented the swelling of the nuclear pellets.

When isotonic saline- CaCl_2 (0.18 mM) without phosphate was used during perfusion of the liver and isolation of the nuclei, and phosphate buffer- CaCl_2 was used only for extraction of the soluble proteins, the patterns (Figure 43C) were substantially like those obtained without phosphate or calcium present. Evidently the presence or absence of phosphate or calcium may determine whether or not a given protein class is brought into solution during isolation or subsequent extraction of the nuclei, but they do not seriously affect the mobility of the proteins after they have been isolated and dialyzed against the electrophoresis buffer. The selective effects obtained using the phosphate buffer with or without calcium should be useful in procedures for isolating individual components.

Figure 43 Moving boundary electrophoresis of soluble nuclear proteins. A. Phosphate buffer used in isolation of nuclei and in extraction of soluble protein. B. Same as A, but with CaCl_2 added to the phosphate buffer. C. Saline- CaCl_2 used in isolation of the nuclei and phosphate- CaCl_2 used in extraction of the soluble protein. Details of procedures are given in the text.

In the standard procedure finally adopted for isolation of the soluble nuclear proteins, the livers were perfused with isotonic saline- CaCl_2 (0.18 mM) and the nuclei were isolated in 75 per cent sucrose- CaCl_2 (0.18 mM). The nuclei were then washed in 30 per cent sucrose and the soluble proteins were extracted with isotonic saline- CaCl_2 (0.18 mM). Washing the isolated nuclei in 30 per cent sucrose minimizes contamination with cytoplasmic components, including glycogen. The presence of calcium chloride in the isolation procedure reduces the tendency of the proteins to precipitate.

From normal animals, this procedure yielded 1.2 ± 0.03 mg soluble protein per mg of DNA. The protein residue remaining after extraction of the soluble proteins and removal of the nucleic acids with hot trichloroacetic acid amounted to 3.5 ± 0.3 mg per mg of DNA. Each experimental value given in this report is the average from determinations on at least five preparations. Typical electrophoresis patterns for material from normal animals are shown in Figure 44, except that it is unusual to see the -0.6 component so prominent in a pattern from normal animals.

The electrophoretic patterns vary appreciably in successive preparations from normal animals, and they change in response to experimental changes in the condition of the animal. Figure 45A shows the pattern obtained from rats starved overnight. Figure 45B shows the pattern for animals of the same group after they had access to food for 2.5 hours.

There is a large increase in the material between +1 and -1. Preliminary experiments have shown that there are distinctive changes in these protein patterns during various altered physiological conditions: recovery from prolonged starvation; liver regeneration after partial hepatectomy; ingestion of thioacetamide or dimethylaminoazobenzene (a liver carcinogen) in the diet.

Figure 44. Moving boundary electrophoresis of soluble nuclear proteins obtained from normal animals by the standard procedure described in the text.

Figure 45. Moving boundary electrophoresis of soluble nuclear proteins. A. Material from animals starved overnight. B. Material from animals in the starved group 2.5 hours after they had access to food once more.

Table 43 gives a summary of the mobilities of electrophoretic components in the nuclear extracts. Each component listed has been observed after two or more different isolation procedures and under two or more physiological conditions, and therefore is not unique to any one set of conditions. The negative mobilities are based on the descending patterns and the positive mobilities on the ascending patterns. The latter actually correspond to descending mobilities since these components are migrating into the protein solution in the ascending limb.

TABLE 43

Mobilities* of electrophoretic components of the soluble nuclear proteins

Class	" +0.8"	" +0.3"	" -0.3"	" -0.6"	" -0.8"	" -1.8"	" -2.2"	" -2.9"	" -5.0"
Mobility	+0.77 $\pm$ 0.08	+0.30 $\pm$ 0.06	-0.30 $\pm$ 0.03	-0.57 $\pm$ 0.04	-0.84 $\pm$ 0.05	-1.83 $\pm$ 0.07	-2.15 $\pm$ 0.09	-2.93 $\pm$ 0.12	-4.95 $\pm$ 0.10

*All mobilities reported are in $(\text{cm}^2 \times 10^5)(\text{V})^{-1}(\text{sec})^{-1}$; each mobility value represents the average and the mean deviation of the results on at least five preparations. The negative mobilities are based on the descending patterns, the positive mobilities on the ascending patterns.

Summary

Studies with the electron microscope have established the ubiquitous presence of pores in the nuclear membrane. There is evidence from many sources indicating that large molecules can and do pass through the nuclear membrane in vivo although this probably represents a process that is controlled and is not simply free diffusion. We have shown that some relatively basic proteins of moderate molecular weight are retained in the nucleus by rather loose ion-binding,^(1,2,6,7) binding so mild that whether they are retained or not may be determined by relatively small changes in the ionic concentration of the medium. We have shown also that the amounts of these proteins retained in the nucleus may change specifically and rapidly with changes in the physiological state of the animal. It is tempting to think that some of these proteins may play a role in the control of cell processes by the nucleus.

References

1. Barton, A. D., and Anna Kane Laird. Progress report: Biochemical studies of cell organelles. Soluble proteins of nuclear and cytoplasmic fractions from rat liver. Biological and Medical Research Division Semiannual Report, January through June 1961, Argonne National Laboratory. ANL-6464, pp. 88-89.
2. Barton, A. D. Soluble proteins of the cell nucleus. The Cell Nucleus, ed. J. S. Mitchell. Butterworth and Co., London, 1960. pp. 142-143.
3. Sorof, S., E. M. Young, and M. G. Ott. Soluble liver "h" proteins during hepatocarcinogenesis by aminoazo dyes and 2-acetylaminofluorene in the rat. Cancer Res. 14, 190-197 (1958).
4. Chaveau, J., Y. Moule, and Ch. Rouiller. Isolation of pure and unaltered liver nuclei. Morphology and biochemical composition. Exptl. Cell Res. 11, 317-321 (1956).
5. Pollister, A. W., and C. Leuchtenberger. The nucleoprotein content of whole nuclei. Proc. Natl. Acad. Sci. U. S. 35, 66-71 (1949).
6. Barton, A. D. Electrophoretic and sedimentation properties of soluble proteins from rat liver cell particulates. Federation Proc. 21, 143 (1962).
7. Barton, A. D., and A. K. Laird. Biochemical and morphological studies of nuclei from rat liver. Biological and Medical Research Division Semiannual Report, January through June 1957, Argonne National Laboratory. ANL-5732, pp. 56-61.

PHOSPHATE ESTERIFICATION AND HYDROLYSIS IN
MITOCHONDRIAL PREPARATIONS OF NORMAL
AND X-IRRADIATED RAT SPLEEN

John F. Thomson

Although the ability of total-body X-radiation to decrease the phosphorylative efficiency of mitochondria isolated from thymus or spleen has been known for several years,^(1,2) there have been a number of questions raised about the significance of these observations. The inability to produce the inhibition by irradiation in vitro,⁽³⁻⁵⁾ the variation in the response with the hormonal state of the animal,⁽⁶⁾ and the failure of this biochemical lesion to develop in mitochondria isolated from livers of irradiated animals⁽⁷⁾ have apparently shown that the impairment of oxidative phosphorylation is not a direct effect of radiation on mitochondria.

The data presented here indicate that there may be no significant lesion at all. The apparent decrease in phosphorylative efficiency is attributable to the presence in the mitochondrial fraction of extraneous materials containing large amounts of phosphatases. When the conditions of assay for oxidative phosphorylation are such that high P/O ratios are observed with mitochondrial preparations from control animals, a negligible effect is seen with preparations from irradiated animals. A decrease in P/O ratios following irradiation is seen only when low P/O ratios are observed with the controls.

Methods

Holtzman or Sprague-Dawley female rats, 7 to 10 weeks old, were used in these experiments. Irradiated rats were killed 4 hours after a dose of 1000 r of X-radiation. Mitochondria were prepared by homogenizing spleens in 9 volumes of 0.25 M sucrose containing 0.0003 M EDTA, removing the nuclei by centrifugation for 10 minutes at 600 x g, and centrifuging the supernatant for 10 minutes at 14,000 x g. The mitochondrial sediment (including the poorly sedimentable layer) was washed once with 0.25 M sucrose containing no EDTA. In some experiments the poorly sedimentable layer was separated by decantation from the more firmly impacted material. In some experiments, a microsome fraction was also isolated by centrifugation for 1 hour at 27,000 x g.

Most of the enzyme assays were carried out according to methods previously employed in this laboratory.⁽⁷⁾ Assays for ATPase and ADPase were carried out in the reaction mixture used for oxidative phosphorylation, with hexokinase and glucose omitted and the nucleotide concentration increased to 0.02 M.

Results

Table 44 shows the results obtained with succinate and α -ketoglutarate as substrates in the oxidative phosphorylation assay. These data were drawn from experiments in which mitochondria, with or without the poorly sedimentable layer, were tested. Most of the experiments were carried out on pooled spleens from four animals. It is clear that there is a negligible effect of radiation on the P/O ratios.

TABLE 44

Oxidative phosphorylation in mitochondria isolated
from spleens of normal and irradiated rats

Substrate	Control			Irradiated		
	No.	P/O*	σ	No.	P/O*	σ
Succinate	24	1.92	0.16	13	1.89	0.15
α -Ketoglutarate	5	2.80	0.28	3	2.76	0.25

*Micromoles P esterified per microatom O consumed.

Table 45 shows the effect of radiation on oxidative phosphorylation in three cytoplasmic fractions: mitochondria, poorly sedimentable layer, and microsomes. These cytologic labels are arbitrary, since the "microsome" fraction contained about 60% of the respiratory activity of the combined fractions. Only the microsome fraction showed an effect of irradiation, but even in this case the difference was not statistically significant.

TABLE 45

Oxidative phosphorylation in different subcellular
fractions of rat spleen mitochondria

Rats	No.	P/O ratios*		
		Mitochondria	Poorly sedimentable layer	Microsomes
Control	6	1.95 $\pm$ 0.09	1.91 $\pm$ 0.19	1.18 $\pm$ 0.28
Irradiated	5	1.88 $\pm$ 0.19	1.81 $\pm$ 0.18	0.89 $\pm$ 0.36

*Averages with standard deviations; substrate, succinate.

The effect of diminishing the concentration of cytochrome c, the usual concentration of which was 12×10^{-6} M, is shown in Table 46. There was a progressive fall in P/O ratios with decreasing concentrations of cytochrome c. The decrease was more pronounced in the irradiated animals; at 1×10^{-6} M cytochrome c the P/O ratio was only half that of the controls, and in the absence of this carrier there was an increase of inorganic phosphate in the medium. (With no cytochrome c added, the oxygen uptake was so low that it could not be measured with the degree of precision suitable for computing a meaningful P/O ratio.) When the differences in the P/N values between the control and irradiated mitochondria at zero cytochrome c concentration are added to the P/N values of the irradiated preparations in order to compensate for hydrolysis, the resultant corrected P/O values are very similar to the controls.

TABLE 46

Effect of cytochrome c concentration on oxidative phosphorylation in rat spleen mitochondria

Concentration of cytochrome <u>c</u>	Control (5 expts.)		Irradiated (3 expts.)		Irradiated, corrected	
	P/N*	P/O**	P/N	P/O	P/N	P/O
10.3×10^{-6} <u>M</u>	35.1	1.86	31.9	1.70	38.1	1.93
5.2×10^{-6} <u>M</u>	27.7	1.88	20.2	1.24	26.4	1.70
2.1×10^{-6} <u>M</u>	14.4	1.70	10.1	1.07	16.3	1.70
1.0×10^{-6} <u>M</u>	10.2	1.63	3.9	0.80	10.1	1.55
0.0×10^{-6} <u>M</u>	3.5	-	-2.7	-	3.5	-

*Micromoles P esterified per mg total N per 20 minutes.

**Micromoles P esterified per microatom O consumed; substrate, succinate.

The results of experiments in which phosphatase activities were studied with several substrates are summarized in Table 47. Oxygen consumption was measured on all samples assayed for phosphatases, and the results are expressed as P(+)/O ratios, the micromoles of phosphate liberated per microatom of oxygen consumed. The most striking differences were obtained with ADP as substrate, although appreciably increased P(+)/O ratios were noted with ATP and glycerophosphate. There was an elevated glucose-6-phosphatase activity in the microsomal fraction, but none in the mitochondria or poorly sedimentable layer. It is probable that there would be little hydrolysis of ATP in the complete system, since in the presence of hexokinase and glucose the concentration of ATP would be extremely low.

TABLE 47

Phosphatase activities of rat spleen mitochondria

Substrate	Control			Irradiated		
	No. rats	P(+)/O*	σ	No. rats	P(+)/O	σ
Adenosine diphosphate	7	0.52	0.21	8	1.49	0.34
Adenosine triphosphate	9	0.46	0.26	9	0.64	0.18
β -Glycerophosphate (acid phosphatase)						
Free	4**	0.67	-	4	0.96	-
Total	4**	2.42	-	4	3.16	-
Glucose-6-phosphate						
Mitochondria	4**	0	-	4	0	-
Poorly sedimentable	4**	0.03	-	4	0.04	-
Microsomes	4**	0.08	-	4	0.12	-

*Micromoles P liberated per microatom O consumed.

**Pooled samples.

Discussion

Four hours after total-body X-radiation considerable destruction of lymphoid cells has taken place. As a result, the preparations of mitochondria isolated from irradiated spleen and thymus contain relatively fewer mitochondria and relatively more lysosomes and microsomes, i.e., there is a diminution of elements capable of esterifying phosphate and an increase in those that hydrolyze phosphate esters.

When the conditions of assay are such as to permit phosphorylation to occur rapidly, the level of ATP liable to hydrolysis is kept low, most of it being bound by the phosphorylation mechanism. However, when phosphorylation is impaired, e.g., by restricting the concentration of cytochrome c, the ADP becomes available to the enzymes with ADPase activity. Since there are relatively larger concentrations of these enzymes in the preparations from irradiated animals, it is not surprising that the P/O ratios are further decreased.

Thus as long as the P/O ratios near the theoretical maximum are obtained with control mitochondria, irradiation will have no effect. When poor P/O ratios are observed in the controls, still lower ones will be observed with irradiated animals because of the relative increase in phosphatase activity. It is noteworthy that previous investigators who had

reported an inhibition of phosphate esterification in hematopoietic tissues of irradiated rats failed to obtain consistently efficient phosphorylation with their control preparations.(1-6,8)

Most of this work was carried out with the technical assistance of Mrs. Jacqueline J. Darling. I wish to thank Dr. Y. E. Rahman for performing the assays for acid phosphatase.

References

1. Potter, R. L. and F. L. Bethel. Oxidative phosphorylation in spleen mitochondria. *Federation Proc.* 11, 270 (1952).
2. Thomson, J. F., W. W. Tourtellotte and M. S. Carttar. Some observations on effect of gamma radiation on the biochemistry of rat thymus. *Proc. Soc. Exptl. Biol. Med.* 80, 268-272 (1952).
3. Van Bekkum, D. W. Oxidative phosphorylation in irradiated tissues. *Chemisch Weekblad* 53, 233-235 (1957).
4. Ord, M. G. and L. A. Stocken. The effects of radiation in vitro on oxidative phosphorylation and adenosine triphosphatase. *Brit. J. Radiol.* 28, 279-282 (1955).
5. Scaife, J. F. and B. Hill. The uncoupling of oxidative phosphorylation by ionizing radiation. *Can. J. Biochem. Physiol.* 40, 1025-1042 (1962).
6. Benjamin, T. L. and H. T. Yost, Jr. The mechanism of uncoupling of oxidative phosphorylation in rat spleen and liver mitochondria after whole-body irradiation. *Radiation Res.* 12, 613-625 (1960).
7. Thomson, J. F. and Y. E. Rahman. Effect of X-irradiation on distribution and properties of cytoplasmic particulates of rat liver. *Radiation Res.* 17, 573-578 (1962).
8. Van Bekkum, D. W. and O. Vos. The effect of X-rays on the oxidative phosphorylation of mitochondria in relation to nuclear damage. *Brit. J. Exptl. Pathol.* 36, 432-438 (1955).

FRAGILITY STUDIES OF RAT SPLEEN LYSOSOMES AFTER X-IRRADIATION

Y. E. Rahman

A recent study shows that rats, after receiving a single dose of 200 r or 1000 r whole-body X-irradiation, selectively retain the cells that contain lysosomes in the spleen and thymus.⁽¹⁾ There is good evidence that lysosomes are mostly, if not exclusively, located in the radioresistant cells of these tissues.^(2,3) Since there is selective retention of lysosomes after irradiation and since the lysosomes contain acid hydrolases which are thought to be involved in cell lysis, one would like to understand the mechanism by which acid hydrolases are released from the boundary membranes of the lysosomes. Thus an attempt was made to investigate possible physical or chemical changes in the membrane of the spleen lysosomes after irradiation and the mechanism involved in these changes.

Methods

Female Holtzman rats, 35 to 45 days old, were used in these experiments. X-irradiation of the animals and preparation of spleen homogenates were essentially the same as described elsewhere.⁽¹⁾ The in vitro irradiation of spleen homogenates was the same, except that the homogenate was kept at 0°C throughout the irradiation period.

Thyrotropic hormone and L-thyroxine were obtained from Sigma Chemical Co. Thyrotropic hormone was administered to the animals subcutaneously at a dose of 2.5 or 5.0 I.U. per 100 g body weight. L-thyroxine was first dissolved in 0.1 N NaOH, and then diluted in 0.25 M sucrose to a final concentration of 1×10^{-5} M.

The method for acid phosphatase assay was the same as described elsewhere.⁽¹⁾

Adrenalectomized and thyroidectomized female Holtzman rats were supplied by Hormone Assay Laboratories. They were given standard post-operative care upon arrival.

Results

Sensitivity of lysosomes to detergent, sucrose, and pH after X-irradiation

Figures 46 and 47 show the release of acid phosphatase from spleen lysosomes of unirradiated and irradiated (1000 r, whole-body) rats treated with non-ionic detergent (Igepal-630) or various concentrations of sucrose

solutions. It can be seen that acid phosphatase was released more readily from spleen lysosomes of irradiated animals than from those of the unirradiated controls.

Figure 46

Igepal-630 concentration vs. acid phosphatase release from spleen lysosomes of irradiated and unirradiated rats. X—X spleen from unirradiated control rats, ●—● 3 hr after 1 kr, ○—○ 24 hr after 1 kr.

Figure 47

Sucrose concentration vs acid phosphatase release from spleen lysosomes of irradiated and unirradiated rats. X—X spleen from unirradiated control rats, Δ—Δ 2 hr after 1 kr, ●—● 24 hr after 1 kr.

Homogenates of spleen from irradiated and unirradiated animals were prepared in 0.25 M sucrose at pH 5, rather than pH 7.4, and incubated at 0°C or 37°C for 30 min. The spleens of irradiated animals had about 30% more free activity than those of the unirradiated controls.

Sensitivity of lysosomes to detergent after 1000 r X-irradiation in vitro

Figure 48 shows clearly that irradiation of spleen homogenates in vitro did not cause increased release of acid phosphatase. This result demonstrates that the effect of X-rays on lysosomes was not due to a direct action on the membranes of the particulates.

Effect of adrenalectomy

Figure 49 shows that X-irradiation had a similar effect on spleen lysosomes of both nonadrenalectomized rats and adrenalectomized rats. Apparently, the increased release of acid phosphatase from spleen lysosomes after X-radiation is independent of the presence of the adrenal glands.

Figure 48

Igepal-630 concentration vs. acid phosphatase release from lysosomes of irradiated and nonirradiated spleen homogenates. x—x spleen from nonirradiated control rats, o—o spleen homogenate irradiated in vitro (1kr).

Figure 49

Igepal-630 concentration vs. acid phosphatase release from spleen lysosomes of irradiated and nonirradiated rats, with and without adrenalectomy. x—x spleen from nonadrenalectomized, nonirradiated control rats, o—o spleen from adrenalectomized, nonirradiated rats, ●—● spleen from adrenalectomized, irradiated rats (3 hr after 1 kr).

Effect of thyroidectomy

It can be seen in Figure 50 that thyroidectomy had the effect of abolishing the difference in acid phosphatase release between irradiated and nonirradiated animals. However, the shape of the acid phosphatase release curve for the nonirradiated thyroidectomized controls was changed.

Figure 50

Igepal-630 concentration vs. acid phosphatase release from spleen lysosomes of thyroidectomized rats, irradiated and nonirradiated. x—x spleen from thyroidectomized, nonirradiated rats, o—o spleen from thyroidectomized, irradiated rats (3 hr after 1 kr), ●—● spleen from thyroidectomized, irradiated rats (24 hr after 1 kr).

Effect of thyrotropic hormone

The effect of thyrotropic hormone on acid phosphatase release was similar to that of irradiation, and the higher dose of hormone was more effective than the lower in producing acid phosphatase release (Figure 51).

Figure 51

Igepal-630 concentration vs. acid phosphatase release from spleen lysosomes of rats given thyrotropic hormone. x—x spleen from control rats, ●—● spleen from rats injected with 2.5 I.U. thyrotropic hormone/100 g body weight, killed 2 hr after injection, ○—○ spleen from rats injected with 5.0 I.U. thyrotropic hormone/100 g body weight, killed 2 hr after injection.

Effect of L-thyroxine in vitro

The effect of L-thyroxine added to spleen homogenate in vitro (Figure 52) resembled that of thyrotropic hormone given to the intact animal (cf. Figure 51).

Figure 52
Igepal-630 concentration vs. acid phosphatase release from spleen homogenates treated with L-thyroxine in vitro. x—x spleen from control rats, ○—○ spleen homogenate treated with 1×10^{-5} M L-thyroxine in vitro for 10 min.

Discussion

The results of these experiments indicate clearly the existence of a relationship between physical (or chemical?) changes of spleen lysosome membranes and the thyroid hormones. Thyroxine has been shown by Tapley^(4,5) and by Lehninger and his co-workers^(6,7) to play a role in the

integrity of the mitochondrial membrane. On the other hand, it is interesting to note that Tata⁽⁸⁾ showed that the pattern of intracellular distribution of endogenous thyroid hormone and that of added tracer radioactive thyroxine in rat liver and skeletal muscle is nonspecific; in other words, there is no preferential localization of these hormones in any particular part of the cells. His results seem to indicate that thyroid hormones act on cell membranes in general, although specificity of this hormonal action on certain types of cells could not be excluded from Tata's experiments since whole tissue homogenates were used.

Botkin et al.⁽⁹⁾ have shown that X-irradiation stimulated the activity of the thyroid gland in rats, and this stimulation could be detected between 2 hours and 30 hours after a dose of 1000 r whole-body X-irradiation. The experimental results of the present study could be explained easily by the indirect effect of X-ray and the direct effect of thyroid hormones on the physical or chemical changes of spleen lysosome membranes.

Studies are under way to find out whether the in vivo thyroid hormonal effect has tissue or organ specificity.

References

1. Rahman, Y. E. Acid-phosphatase and β -glucuronidase activities of thymus and spleen of rats after whole-body x-irradiation. *Proc. Soc. Exptl. Biol. Med.* 109, 378-381 (1962).
2. Rahman, Y. E. Electron microscopy of lysosome-rich fractions from rat thymus isolated by density-gradient centrifugation before and after whole-body x-irradiation. *J. Cell Biol.* 13, 253-260 (1962).
3. Balner, H., L. J. Old and D. A. Clarke. Reduction of x-ray lethality in mice by agents affecting the reticuloendothelial system. *Radiation Res.* 15, 836-849 (1961).
4. Tapley, D. F. The effect of thyroxine and other substances on the swelling of isolated rat liver mitochondria. *J. Biol. Chem.* 222, 325-339 (1956).
5. Tapley, D. F. The mechanism of action of thyroid hormones. *Am. Zoologist* 2, 373-378 (1962).
6. Lehninger, A. L., B. L. Ray and M. Schneider. The swelling of rat liver mitochondria by thyroxine and its reversal. *J. Biophys. Biochem. Cytol.* 5, 97-108 (1959).

7. Lehninger, A. L. and B. L. Ray. Action of thyroxine on mitochondria. *Science* 125, 748-749 (1957).
8. Tata, J. R. Intracellular and extracellular mechanisms for the utilization and action of thyroid hormones. *Recent Progress in Hormone Research* 18, 221-259 (1962).
9. Botkin, A. L., E. H. Praytor, M. E. Austing and H. Jensen. Thyroid response to total body x-irradiation. *Endocrinology* 50, 550-554 (1952).

SPONTANEOUS NON-LETHAL DAMAGE IN A "SMALL" CLONE OF CHINESE HAMSTER CELLS IN CULTURE

Warren K. Sinclair and Carolan H. Wieker*

A study of X-ray-induced non-lethal damage in Chinese hamster cells, which is expressed in the form of small colonies, increasing in frequency with X-ray dose,⁽¹⁾ posed the question whether such damage occurred spontaneously in the unirradiated culture. This possibility could be established if, from a normal unirradiated cell population, one could isolate a small colony moiety that exhibited the same features as irradiated small colonies, namely, slow growth, poor plating efficiency and enhanced radiosensitivity. In addition, these clones are sometimes polyploid, whereas the normal line is mainly diploid. Therefore, attempts were made to isolate, from a normal population of Chinese hamster lung cells grown in culture, a clone that would give rise to a subline possessing the above properties. A slow-growing clone, probably with large cells indicating polyploidy, seemed most likely to show these properties.

Materials and Methods

Culture of normal cell line

The cell line was grown from samples of clone V79-1 of Chinese hamster lung tissue originally cultured by Ford and Yerganian⁽²⁾ and supplied to one of us (WKS) in May 1961 by M. M. Elkind of the National Cancer Institute. Cell populations were cultivated on 85-mm plastic petri dishes (Falcon Plastics, Los Angeles), using a medium similar to the HUT-15 of Elkind and Sutton,⁽³⁾ and were incubated at 37°C in a humid atmosphere of 2% CO₂ and air. Single-cell suspensions were obtained by trypsinizing cultures growing in log phase with 4 ml of 0.03% trypsin (1:300) in saline for 15 minutes. About 99% of the normal population cells were then single.

Subline

The subline was established by a two-step clonal isolation. Approximately 50 cells from the normal line were inoculated into each of five plates and incubated for 14 days. The plates were scanned and of the very few small clones present several were chosen for isolation. Each clone was surrounded by a glass tube sealed to the bottom of the plate and the cells of the clone were trypsinized from within the tube. The growth rate of each of the new populations obtained was, however, not much slower than that of the parent line (doubling time, D.T., ~10-11 hours). Therefore, after a further 18 days, two small clones were isolated from these new populations and each was divided into six aliquots. One of these aliquots appeared to grow slowly

---

* Argonne Semester Student from Coe College.

(D.T. ~24 hours, estimated by microscopic observation), and the population appeared to be homogeneous and to consist of rather large cells. This population was cultivated and became the subline of Experiment 70.

Studies on the subline

In Experiment 70, the subline population growing in log phase was trypsinized to provide 98% single cells (more than 20 minutes' trypsinization were required for this). This suspension was used to inoculate vessels as follows: a) fresh plates were inoculated with various cell concentrations (ranging from 320 to 140,000 cells per plate) for X-ray survival and plating efficiency (P.E.) studies; b) plates were inoculated with a constant number of cells (about 15,000 cells per plate) for growth-rate and cell size distribution studies; c) two bottles were inoculated with about 10^6 cells for chromosome analysis.

Growth studies and cell size

The growth rate of the subline was found by trypsinizing two of the incubated plates daily to provide a single-cell suspension the concentration of which was determined on a Coulter counter set at Amp. 1., Ap. C. 8., and L.T. 12. The cell population size distribution was found from the same suspensions using the Coulter counter and size distribution plotter set at Amp. 1., Ap. C. 8., L.T. 12, U.T. 50, Scale 6 and 4 seconds per channel.

Chromosome analysis

The day after inoculation Colcemide was added to the bottle cultures to arrest mitoses at metaphase. Two hours later chromosome spreads were made by the air-dry method of Moorehead,⁽⁴⁾ modified by Stroud et al.⁽⁵⁾ for tissue culture cells. The slides were stained with aceto-orceine and examined at 1250 x under a phase microscope. The chromosome number of 52 metaphase figures was recorded.

X-ray survival

The plates were inoculated to yield about 200 surviving clones per plate, requiring inocula of from about 320 to 140,000 cells. The plates were incubated for 2 hours after inoculation and then exposed to one of 9 X-ray doses ranging from 0 to 1500 r. The doses were delivered at 110 r/min (measured by a Victoreen ion chamber) from a 250 kvp X-ray machine with H.V.L. 0.9 mm Cu. The absorbed dose rate was found, by ferrous sulfate dosimeter, to be 0.945 rads/r. Plates were out of the incubator for about 20 minutes during exposure and thereafter additional medium was added to the 10 ml of medium already on the plates, for the higher inoculations. After 12 days of incubation the plates were stained with 1.5% methylene blue, and the colonies were counted on a bacterial colony counter.

Results

From the growth curve of this subline, shown in Figure 53, a doubling time of 24 hours (more than twice the normal value) was found.

Figure 53. Growth of subline population

Cell size distributions varied slightly throughout the growth period, but all showed a cell size peak about twice that of normal cells (Figure 54). This type of size distribution is characteristic of tetraploid cells.^(1,6) The chromosome study (Figure 55) showed many polyploid elements in the subline, with a modal number at 44, in the tetraploid region, as compared with the normal diploid population, with modal number at 22 or 23.

The X-ray survival data are shown in Figure 56. The curve fits the multitarget relation $S = 1 - (1 - e^{-D/D_0})^n$, where S is the surviving fraction, D the dose in rads, n the target number (found by extrapolation), and D_0 the average inactivation dose in rads (the reciprocal of the slope of the curve). For this subline, the data of Figure 56 indicate $n \simeq 4$, $D_0 \simeq 130$ rads, as compared with $n = 2.5-4.5$ and $D_0 = 200-210$ rads for the parent line.⁽¹⁾ This subline is, therefore, considerably more sensitive to X-radiation than the parent line, but it has about the same sensitivity as many of the small colonies induced by X-radiation at 1500 r.

The plating efficiency of the subline is low, about 25%, as compared with values for the normal population which vary between 60% and 90%.

Figure 54. Cell size distribution of (a) polyploid subline (b) normal hamster population obtained with the same settings on the Coulter counter (Amp. 1., Ap. C. 8, L.T. 12, M.T. 50, Scale 6, 4 sec. per channel).

Figure 55. Chromosome number distribution of subline population

Figure 56. X-ray survival of subline population

Discussion

This unirradiated subline appears to exhibit the same features as small colonies induced by 1500 r of X-radiation; thus, the damage induced by X-radiation may also arise spontaneously. The natural occurrence is rare, however, since the isolation of this subline required selecting a fraction following two clonal choices for the smallest colony on the plate.

Later studies have shown that the polyploidy of this subline is not a unique feature of all sublines developed from small colonies. Irradiated small clones, only slightly changed in chromosome number from the normal diploid,^(1,6) show the same properties. Furthermore, fast growing tetraploids isolated from the parent line have normal radiosensitivity. Radiosensitivity, therefore, appears to be associated with slow growth and poor plating efficiency but is not necessarily uniquely determined by them.

The subline discussed here maintained similar properties over a period of more than 5 months, with at most a very slight recovery to D.T. $\approx$ 20 hours, $n \sim 5$, $D_0 \approx 155$ rads and P.E. of 34%. Presumably, normal properties can only be recovered in such a subline if the population is capable of giving rise to normal progeny.

The technical assistance of Sonia Swanson, Grace Racster and Beverly Svoboda is gratefully acknowledged.

References

1. Sinclair, W. K. X-ray induced heritable damage (small colony formation) in cultured mammalian cells. Radiation Res., in press.
2. Ford, D. K. and G. Yerganian. Observations on the chromosomes of Chinese hamster cells in tissue culture. J. Natl. Cancer Inst. 21, 393-425 (1958).
3. Elkind, M. M. and H. Sutton. Radiation response of mammalian cells grown in culture. I. Repair of X-ray damage in surviving Chinese hamster cells. Radiation Res. 13, 556-593 (1960).
4. Moorehead, P. S., P. C. Nowell, W. J. Mellman, D. M. Battips, and D. A. Hungerford. Chromosome preparations of leukocytes cultured from human peripheral blood. Exptl. Cell Res. 20, 613-616 (1960).
5. Stroud, A. N., A. M. Brues, and B. R. Svoboda. DNA synthesis of mammalian kidney cells in tissue culture after single and periodic doses of irradiation. Ann. N. Y. Acad. Sci. 95, 942-949 (1961).
6. Sinclair, W. K. and A. N. Stroud. Chromosome number and aberrations in "large" and "small" clones of irradiated Chinese hamster cells in culture. This report, p. 161.

CHROMOSOME NUMBER AND ABERRATIONS IN "LARGE" AND "SMALL" CLONES OF IRRADIATED CHINESE HAMSTER CELLS IN CULTURE

Warren K. Sinclair and Agnes N. Stroud

Many cultured mammalian cell lines, when examined by the cloning technique of Puck⁽¹⁾ after exposure to a range of doses of X-radiation, show a broadening of the colony size spectrum due to an increase in the number of small colony survivors as the dose increases. In the Chinese hamster cell line used in this investigation, after 1500 r of X-radiation, large colonies are found to have normal growth rates, plating efficiencies and radiosensitivity. Small colonies, however, appear to suffer from a form of heritable damage which manifests itself in slow growth, poor plating efficiency and enhanced radiosensitivity.⁽²⁾ The present study was undertaken to determine whether changes in chromosome number or the frequency or type of chromosome aberrations are associated with the damage observed in small colony survivors.

Materials and Methods

Experiments were conducted with cells of a Chinese hamster line derived from female lung tissue, strain V, clone V79-1,^(3,4) incubated on 85-mm plastic petri dishes using medium similar to the HUT-15 of Elkind and Sutton,⁽⁴⁾ at 37°C in a humid atmosphere of 2% CO₂ and air. Radiation survival data were obtained by exposing plates of different inocula to doses up to 1500 r of 250 kvp X-radiation (HVL 0.9 mm Cu; dose rate 110 r/minute), to provide 100-200 colonies per plate. Medium was added to the higher inoculum plates immediately after exposure, and the plates were incubated for 12 to 14 days before staining with methylene blue for colony counting. Survival data were analyzed according to the relation

$$S = 1 - \left(1 - e^{-D/D_0}\right)^n$$

where

S = fractional survival,

D = dose (rads),

D₀ = dose which reduces S by a factor of 0.37 on the exponential part of the curve, and

n = extrapolation number.

For the normal stem line of diploid Chinese hamster cells the survival curve of Figure 57, for which $n = 2.5$, $D_0 = 203$ rad, is typical. The plating efficiency was regularly 60% to 80% for 99% single cells. The dose was

Figure 57. Survival of Chinese hamster cells after 250 kVp X-radiation. O, Exp. 30; X, Exp. 32. For

$$S = 1 - (1 - e^{-D/D_0})^n, D_0 = 203 \text{ rad and } n = 2.46 \text{ for this curve.}$$

measured with a Victoreen chamber at the level of the cells, and the absorbed dose was checked with ferrous sulfate solution. (The absorbed dose was found to be 0.945 times the exposure dose, i.e., 0.945 rad/r.) Small colony frequency increases with increasing dose,⁽²⁾ and at 1500 r, survival $\sim 0.2\%$, small colonies predominate (Figure 58). From plates with fewer survivors, after 1500 r, than that of Figure 58, colonies of various sizes were isolated by surrounding them with a glass tube and trypsinizing only the cells within the tube. New populations, or sublines, were grown out from these isolated colonies (and in some cases from as many as ten aliquots of a colony) for subsequent study. Colonies were selected as follows: large, 3-4 mm diameter, 20,000-100,000 cells; medium, 1-2 mm diameter, 1000-5000 cells; small, <1.0 mm diameter, 40-400 cells. All colonies within these size ranges, incubated for as long as 14 days, grew successfully on transplantation. The sub-

lines were maintained by transferring both in dishes and in bottles after 7 to 10 generations.

Sufficient cells were available for study 1 to 5 weeks after isolation, i.e., 3 to 7 weeks after X-irradiation. Cell size distributions, growth rate and radiation survival were then studied.⁽²⁾ At the same time, about 10^6 cells were inoculated into each of two 3-oz. prescription bottles for chromosome assay. The day following inoculation Colcemide was added to the bottle cultures to arrest mitoses at metaphase. Two hours later, chromosome spreads were made by the air-dry method of Rothfels et al.⁽⁵⁾ modified by Stroud et al.⁽⁶⁾ for tissue culture cells. The slides were stained with aceto-orcein and examined under a phase microscope at 1250X magnification.

Figure 58. Colonies of Chinese hamster cells, Exp. 30, stained with methylene blue, 13 days after inoculation

Results

Large clones were found to grow at the same rate as the normal population, (doubling time, D.T., about 10-12 hr), small clones at a much slower rate (D.T., 20-35 hr) and medium clones at intermediate rates (D.T., 14-20 hr), Figure 59. Small clones also have poor plating efficiency and enhanced radiosensitivity (Figure 60); the values of n (5) and D_0 (147 rad) are typical of many small colonies.⁽²⁾

Cell size distribution and chromosome number

Cell size distributions for a selection of irradiated clone sublines are compared with the normal population (33-0) in Figure 61. These distributions were obtained at constant settings on the Coulter counter and size distribution plotter. The peak of the curve represents the modal size for the population. Additional data on cell size peaks for other sublines using the same instrument settings are given in Table 48. The chromosome data for a number of colonies in each category are also shown in Table 48. Chromosome number distributions appear to be broadened for most, if not all, irradiated clones. Sometimes the change is slight, for example, in large clones 33-III A and 71, and small clones 39I3, but in other cases the change is very marked, for example, in large clone 72, small clones 44 and 80. In some cases, for example, in small clone 33IA, the chromosome distribution is mainly subdiploid and the size peak is smaller than normal. Complete chromosome distributions for the same sublines for which size distributions are shown in Figure 61 are shown in Figure 62. Additional modal chromosome numbers are given in Table 48.

Figure 59

Growth rates of Chinese hamster clones after 1500 r. Large clones after 1500 r have the same growthrate as the normal population.

Figure 60
Survival of normal population (Exp. 48)
and small colony population (Exp. 49)
after 250 kvp X-radiation

Figure 61 Cell size distributions for normal and irradiated clones obtained with a Coulter counter and size distribution plotter set at AMP. 1, Ap. C8, LT 12, UT 50, Scale 6.

TABLE 48

Characteristics of several colonies in each size category

Colony type	Expt. No.	Days after irradiation	No. cells observed	Modal chromosome No.	Cell size peak	Per cent abnormal cells	Per cent abnormal type		
							Chromatid	Isochromatid	Exchanges
Normal	33-0 0-6	-	40	22	7-8	2.5	100	-	-
		-	52	23	8-9	5.8	100	-	-
Large	33III A	20	44	22-23	8	9.1*	25	-	75
	71	21	52	23	7	3.8	100	-	-
	72	22	50	39-42	12*	2.0	-	-	100
Medium	33II A	21	39	22	8-10	20.5	-	12.5	87.5
	33II B	20	32	22	8	6.3	100	-	-
Small	33IA	27	52	18-22	5-6	1.9	-	-	100
	39I3	45	40	22	7	2.5	100	-	-
	42	54	42	22-24	8-9	21.4*	11.1	22.2	66.7
	44	55	42	36-40	13-15	31.0*	53.8	-	46.2
	49	80	32	34-40	12-13	6.3	100	-	-
	53	91	30	37-40	12-14	6.7	50	-	50
	54	101	52	40	13-14	3.8	50	-	50
	58	119	44	37-40	12	2.2	-	-	100
	64	147	36	37	13-14	2.8	-	-	100
	79	42	64	22	7	12.5	-	-	100
	80	43	50	50	11-15	18.0	23.0	7.7	69.3
	86	55	56	21-22	7	1.8	-	-	100
Reirradiated	55	55	74	33-36	12-14	2.7	50	-	50
Unirradiated	70	-	52	44	15-16	3.8	25	-	75

* These sublines also contain many 'minutes.'

† Experiment 44.

Figure 62

Distribution of chromosome number per cell in the normal population and in clones after 1500 r X-radiation

There is clearly no correlation between chromosome number and colony size. Any of the chromosome distributions may occur in any size category of clone. However, the chromosome number distribution correlates very well with the cell size distribution. Normal or near normal diploid populations have modal peaks at channels 7-9 on the Coulter counter distribution plotter with the settings indicated; slightly broadened chromosome distributions have cell size modal peaks at channels 8 to 11, subdiploids at channels 5 to 7, and modal tetraploids have peaks between channels 12 and 17, with many large cells.

Furthermore, the ratio of DNA to protein was measured in samples from the normal diploid population, the irradiated tetraploid of Exp. 44, and the unirradiated tetraploid of Exp. 70. This ratio was found to be almost the same for each, with values between 0.07 and 0.09. The cell volume might be expected to be nearly proportional to DNA content for these cell lines, and this appears to be the case.

Chromosome aberrations

The number of cells examined and the number of aberrations observed in many of the sublines are too few for detailed conclusions to be established. However, there are some definite trends in the data of Table 48. The unirradiated populations 33-0, 0-6, and 70 showed few abnormalities and all of these were of the chromatid type. No isochromatid breaks or exchanges were observed. In almost all of the irradiated clones isochromatid breaks, or exchanges, or both were found.

In scoring chromatid breaks, deletions were included but 'gaps' were not. The latter were excluded because of the difficulty of determining whether they were true breaks or not. However, since these occurred indiscriminately in both large and small clones, and their inclusion would affect the numbers of cells with abnormalities in Table 48 by a few per cent in some cases, neither the trend of the data nor the arguments to follow would be affected. Gaps usually were found at the site of secondary constriction in the chromosomes. Both deletions and gaps occurred most frequently in the large metacentric chromosomes I, II, and III; a few occurred in the subtelocentrics. For a more detailed discussion of the classification of chromosomes of this stem line, see Reference 7. The percentage of cells with abnormalities in the three large-clone sublines examined was not high, but exchanges had become apparent.

Among the seven small clone sublines studied the percentage of cells with aberrations varied. Three had very low values, two had moderate values and two had high values. The highest, Exp. 44, was actually an aliquot (0.1) of a small colony which grew out very slowly and thus was a highly selected fraction of a small colony. At the time of examination, the large-clone sublines had passed through more cell generations, up to 50, than most of the

small-clone sublines (~30 generations), and, as the performance of the Exp. 44 subline (Exps. 44-64) shows, chromosome abnormalities appear to be deleted with successive transfers and generations. Thus, the higher percentage of abnormalities found in a few of the small clones may also be present in the large clones at an earlier stage. In any case, there is no clear correlation here between the percentage of abnormalities and the clone type of origin.

It may be noted that between small clones with a high proportion of abnormalities (e.g., 44) and those with a low proportion (e.g., 86) no differences in growth rate, plating efficiency, or radiosensitivity were evident. Furthermore as the six successive experiments (44-64) on one subline show, there is no change in the properties of the subline while the abnormalities are being deleted (Table 49). There is also no change in chromosome number distribution (Figure 63).

TABLE 49

Successive transfers of small colony subline

	Experiment						Normal
	44	49	53	54	58	64	
Growth							
Doubling time, hr	29	26	26	28	29.5	25	10.8
Cell size							
Distribution peak	13-15	12-13	12-14	13-14	12-13	12	7-8
X-ray survival							
Plating efficiency, %	29	36	23	37	24	30	70
Hit number, n	4.18	5.05	5.84	4.98	3.54	4.8	3-4
Slope D ₀ , rads	135*	147	146	140	156 †	145	205
Chromosomes							
Modal peak	36	34	37	40	37	37	22
	<u>40</u>	<u>40</u>	40 72	72	40		
Aberrations (total), %	31	6.3	6.7	3.8	2.2	2.8	2.5

* Inocula not very suitable.

† Some plates lost.

Figure 63. Chromosome numbers per cell for successive transfer of a small colony (after 1500 r) over a period of 4 months

The frequency of aberrations does not appear to be correlated either with colony type or with chromosome number distribution.

Reversion of clones to normal

Some of the radiation-damaged clones, especially those arising from medium colonies, appear to revert nearly to normal (in regard to such properties as growth, plating efficiency, and radiosensitivity) after a few transfers. Some lose their aberrations at the same time, but the chromosome number distribution remains essentially unchanged. These other properties of the cell population appear to be substantially unaffected by their chromosome complement. Some of the small colonies, e.g., the sublines of Exp. 44 and 79, have shown no reversion for as long as 1 year. Presumably no elements in such a subline are capable of giving rise to normal progeny.

Discussion

Heteroploidy and aberrations appear to occur quite frequently in cell populations grown out from colonies originally exposed to 1500 r. However, neither the heteroploidy nor the aberration frequency appears to be correlated with the size of the original colony - small, medium or large. The heritable damage manifested as a small colony survivor, which appears to result in slow growth, poor plating efficiency, and enhanced radiosensitivity, does not seem to be associated with observable chromosome changes in the cell.

We are indebted to Dr. A. D. Barton for the assay of DNA/protein ratio.

References

1. Puck, T. T., P. I. Marcus and S. J. Cieciura. Clonal growth of mammalian cells in vitro. J. Exptl. Med. 103, 273-283 (1956).
2. Sinclair, W. K. X-ray induced heritable damage (small colony formation) in cultured mammalian cells. Radiation Res., in press.
3. Ford, D. K. and G. Yerganian. Observations on the chromosomes of Chinese hamster cells in tissue culture. J. Natl. Cancer Inst. 21, 393-425 (1958).
4. Elkind, M. M. and H. Sutton. Radiation response of X-ray damage in surviving Chinese hamster cells. Radiation Res. 13, 556-593 (1960).
5. Rothfels, K. H. and L. Siminovitch. An air-drying technique for flattening chromosomes in mammalian cells grown in vitro. Stain Technol. 33, 73-77 (1958).
6. Stroud, A. N., A. M. Brues and B. R. Svoboda. DNA synthesis of mammalian kidney cells in tissue culture after single and periodic doses of irradiation. Ann. N. Y. Acad. Sci. 95, 942-949 (1961).
7. Yu, C. K. Spontaneous chromosome aberrations and the nature of near-diploid cells of Chinese hamster in vitro. This report page 171.

SPONTANEOUS CHROMOSOME ABERRATIONS AND THE NATURE
OF NEAR-DIPLOID CELLS OF CHINESE HAMSTER
(CRICETULUS GRISEUS) IN VITRO

C. K. Yu

Introduction

It is well known that chromosome aberrations occur spontaneously in the cells of long-term cultures in vitro. The chromosome aberrations of Chinese hamster cells grown in culture usually are found to be either chromatid or chromosomal breaks, leading to the formation of abnormal chromosomes. Chromosome morphology and the number of chromosomes may, therefore, vary from cell to cell. Normal diploid Chinese hamster cells have 22 chromosomes. Each pair of chromosomes has been assigned a number according to its entire length, size, and the position of the centromeres.⁽¹⁾

After long-term cultivation in vitro, the number of chromosomes of Chinese hamster cells has been found to be near-diploid, ranging between 20 and 26, with the majority of cells having 23. There is a small percentage of polyploidy in the population.⁽²⁾

The morphological relationship among the chromosomes in these near-diploid cells has not been described. It would be of interest to know, first, whether these near-diploid cells have similar chromosome composition, that is, whether cells containing the same number of chromosomes actually possess the same component chromosomes and whether cells containing different numbers of chromosomes have similar chromosome patterns except for the addition of one or two extra chromosomes (fragments). Second, if the cells are abnormal in chromosome constitution, it would be of interest to know whether the chromosomal aberrations occur at specific regions of one chromosome or at nonspecific regions of any chromosome. In this report I have attempted to answer these questions.

Materials and Methods

The cell line of Chinese hamster (Cricetulus griseus) used in this study has been cultured by W. K. Sinclair⁽³⁾ on plastic Petri dishes, using HUT-15 medium similar to that of Elkind and Sutton.⁽⁴⁾ The cells were incubated at 37°C in a humid atmosphere of 2% CO₂ and air. This cell line was originally cultured from lung tissue and designated V strain by Ford and Yerganian;⁽¹⁾ it was subcultured by M. M. Elkind (clone V79-1) in December, 1958, and has been maintained in this laboratory since May, 1961.

Cells for chromosome analysis were harvested at log-phase growth stage. The preparation of chromosomes was the same as described by Hsu and Klatt⁽⁵⁾ with the following modifications: cells were treated with 0.3 mg/ml trypsin at 37°C for 15 minutes, centrifuged at 500 rpm for 5 minutes, pretreated for 30 minutes in hypotonic (0.9%) sodium citrate solution, fixed for one hour in acetic-alcohol (1:3), and stained with 2% natural orcein in 50% acetic acid. No colchicine pretreatment was used. Microscope slides were sealed with Krönig wax and examined under the Zeiss phase-contrast microscope.

Chromosome studies were made of 100 metaphase cells. However, a much larger number of slides had to be prepared to provide enough metaphase cells for analysis because no colchicine was used for pretreatment. The application of this agent, although useful in providing abundant metaphase cells, causes the chromosomes to contract, making detailed comparisons of chromosome morphology very difficult.

Chromosomes were cut out of photographs of metaphase cells and matched in a line for comparison. The actual lengths of chromosomes and the ratios between the lengths of the two arms were measured with a micrometer, the scale of which was photographed and developed on transparent film at various magnifications. After measurement the chromosomes were classified according to the nomenclature of Ford and Yerganian.⁽¹⁾ When there was no difficulty in identification, chromosomes were assigned Roman numerals in the usual way; these are called "normal" chromosomes. However, in each of the cells there were several chromosomes whose origin could not be identified; these are called "extra" chromosomes.

Results

Number of chromosomes

The majority of the cells (69%) contained 23 chromosomes; however, chromosome number ranged between 20 and 26 (Figure 64). In addition, about 2% of the mitotic cells in the population were found to be in the tetraploid range. No normal (classical) diploid cells were found in this population. The cells having 22 chromosomes were abnormal in chromosome constitution, because of the absence of several normal chromosomes and the presence of several extra chromosomes (Table 50).

Chromosome constitution

In the near-diploid cells, only one homologue of a pair was absent, i.e. the cells still had at least one homologous chromosome of the pair normally present (Figures 65-80). Although chromosome aberrations, such as chromatid or chromosomal breaks, or deletions, have sometimes been seen in "normal" chromosomes, these chromosomes can still be

Figure 64

Distribution of chromosome number of 100 metaphase near-diploid cells of Chinese hamster V cell strain in vitro

TABLE 50

Chromosome complement of 100 metaphase cells of Chinese hamster V cell strain in vitro

Chromosome count	Cell count	Chromosome #I	Chromosome #II	Chromosome #XIII	Chromosome #III	Chromosome #IV	Chromosome #V	Chromosome #VI	Chromosome #VII	Chromosome #IX	Chromosome #X	Chromosome #XI	Extra chromosomes		
													Metacentric	Submetacentric	Telocentric subtelocentric
26	2	2	2	1	2	1	1	1	1	1	1	1	1	3	2
21	1	2	2	1	2	1	1	1	1	1	1	1		2	5
21	1	2	2	1	2	1	1	2	1	1	2	1		2	2
21	1	2	2	1	1	2	1	1	1	1	2	1		2	4
21	1	2	2	1	1	2	2	1	1	2	1	1		3	2
22	2	2	2	1	2	1	1	1	1	1	1	1		1	7
22	4	2	2	1	2	1	1	2	1	2	1	1		2	4
22	4	2	2	1	2	1	1	2	1	1	2	1		2	4
22	1	2	2	1	1	2	1	1	1	1	2	1		2	5
22	1	2	2	1	2	2	1	1	1	2	2	1		2	3
22	1	2	2	1	2	2	1	2	1	2	2	1		1	3
23	1	2	2	1	2	1	1	1	1	1	1	1		1	8
23	25	2	2	1	2	1	1	1	1	1	2	1		1	7
23	26	2	2	1	2	1	1	1	1	1	2	1		2	6
23	1	2	2	1	2	1	1	1	2	1	2	1		1	6
23	1	2	2	1	2	1	1	2	1	1	2	2		2	3
23	4	2	2	1	2	1	1	1	1	2	1	1		3	5
23	2	2	2	1	2	1	1	2	1	2	1	1	1	2	4
23	1	2	2	1	2	1	1	1	1	2	2	1		2	5
23	2	2	2	1	2	1	1	1	2	2	2	1		2	5
23	1	2	2	1	1	2	1	1	1	2	1	1		2	6
23	1	2	2	1	1	2	1	1	1	2	2	1		3	4
23	1	2	2	1	1	2	1	1	2	2	1	2		1	5
23	1	2	2	1	2	2	1	1	1	1	2	2		1	5
23	1	2	2	2	1	1	1	1	1	2	2	1		1	6
23	1	2	2	2	2	1	1	2	1	2	2	1		1	4
24	2	2	2	1	2	1	1	1	1	1	1	1		3	7
24	1	2	2	1	2	1	1	1	1	1	2	1		2	8
24	3	2	2	1	2	1	1	1	1	1	2	1		2	7
24	1	2	2	1	2	1	1	1	1	2	1	1		2	7
24	2	2	2	1	2	1	1	2	1	2	1	1		2	6
24	1	2	2	2	2	1	1	2	1	2	2	1		1	5
25	1	2	3	2	2	2	1	1	1	1	2	1		2	5
26	1	2	3	2	2	2	1	2	1	2	2	2		0	5

Figure 65. Pattern A: a cell with 20 chromosomes; 14 normal and 6 extra chromosomes.

Figure 66. Pattern A: a cell with 21 chromosomes; 14 normal and 7 extra chromosomes.

Figure 67. Pattern A: a cell with 22 chromosomes; 14 normal and 8 extra chromosomes.

Figure 68. Pattern A: a cell with 23 chromosomes; 14 normal and 9 extra chromosomes.

Figure 69. Pattern A: a cell with 24 chromosomes; 14 normal and 10 extra chromosomes.

Figure 70. Pattern B: a cell with 21 chromosomes; 16 normal and 5 extra chromosomes.

Figure 71. Pattern B: a cell with 22 chromosomes; 16 normal and 6 extra chromosomes.

I	II	VIII	III	IV	V	VI	VII	IX	X	XI	EXTRA CHROMOSOMES N. TACONIA 1946 EW. TACONIA 1946

Figure 72. Pattern D: a cell with 23 chromosomes; 15 normal and 8 extra chromosomes (1 submetacentric with 7subtelocentrics and telocentrics).

Figure 73. Pattern D: a cell with 24 chromosomes; 15 normal and 9 extra chromosomes (1 submetacentric with 8subtelocentrics and telocentrics).

Figure 74. Pattern D: a cell with 23 chromosomes; 15 normal and 8 extra chromosomes (2 submetacentrics with 6 subtelocentrics and telocentrics).

Figure 75. Pattern D: a cell with 24 chromosomes; 15 normal and 9 extra chromosomes (2 submetacentrics with 7 subtelocentrics and telocentrics).

Figure 76. Pattern E: a cell with 22 chromosomes; 16 normal and 6 extra chromosomes.

Figure 77. Pattern E: a cell with 23 chromosomes; 16 normal and 7 extra chromosomes.

Figure 78. Pattern E: a cell with 24 chromosomes; 16 normal and 8 extra chromosomes.

Figure 79. Pattern F: a cell with 21 chromosomes; 15 normal and 6 extra chromosomes.

Figure 80. Pattern F: a cell with 22 chromosomes; 15 normal and 7 extra chromosomes.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144	145	146	147	148	149	150	151	152	153	154	155	156	157	158	159	160	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180	181	182	183	184	185	186	187	188	189	190	191	192	193	194	195	196	197	198	199	200	201	202	203	204	205	206	207	208	209	210	211	212	213	214	215	216	217	218	219	220	221	222	223	224	225	226	227	228	229	230	231	232	233	234	235	236	237	238	239	240	241	242	243	244	245	246	247	248	249	250	251	252	253	254	255	256	257	258	259	260	261	262	263	264	265	266	267	268	269	270	271	272	273	274	275	276	277	278	279	280	281	282	283	284	285	286	287	288	289	290	291	292	293	294	295	296	297	298	299	300	301	302	303	304	305	306	307	308	309	310	311	312	313	314	315	316	317	318	319	320	321	322	323	324	325	326	327	328	329	330	331	332	333	334	335	336	337	338	339	340	341	342	343	344	345	346	347	348	349	350	351	352	353	354	355	356	357	358	359	360	361	362	363	364	365	366	367	368	369	370	371	372	373	374	375	376	377	378	379	380	381	382	383	384	385	386	387	388	389	390	391	392	393	394	395	396	397	398	399	400	401	402	403	404	405	406	407	408	409	410	411	412	413	414	415	416	417	418	419	420	421	422	423	424	425	426	427	428	429	430	431	432	433	434	435	436	437	438	439	440	441	442	443	444	445	446	447	448	449	450	451	452	453	454	455	456	457	458	459	460	461	462	463	464	465	466	467	468	469	470	471	472	473	474	475	476	477	478	479	480	481	482	483	484	485	486	487	488	489	490	491	492	493	494	495	496	497	498	499	500	501	502	503	504	505	506	507	508	509	510	511	512	513	514	515	516	517	518	519	520	521	522	523	524	525	526	527	528	529	530	531	532	533	534	535	536	537	538	539	540	541	542	543	544	545	546	547	548	549	550	551	552	553	554	555	556	557	558	559	560	561	562	563	564	565	566	567	568	569	570	571	572	573	574	575	576	577	578	579	580	581	582	583	584	585	586	587	588	589	590	591	592	593	594	595	596	597	598	599	600	601	602	603	604	605	606	607	608	609	610	611	612	613	614	615	616	617	618	619	620	621	622	623	624	625	626	627	628	629	630	631	632	633	634	635	636	637	638	639	640	641	642	643	644	645	646	647	648	649	650	651	652	653	654	655	656	657	658	659	660	661	662	663	664	665	666	667	668	669	670	671	672	673	674	675	676	677	678	679	680	681	682	683	684	685	686	687	688	689	690	691	692	693	694	695	696	697	698	699	700	701	702	703	704	705	706	707	708	709	710	711	712	713	714	715	716	717	718	719	720	721	722	723	724	725	726	727	728	729	730	731	732	733	734	735	736	737	738	739	740	741	742	743	744	745	746	747	748	749	750	751	752	753	754	755	756	757	758	759	760	761	762	763	764	765	766	767	768	769	770	771	772	773	774	775	776	777	778	779	780	781	782	783	784	785	786	787	788	789	790	791	792	793	794	795	796	797	798	799	800	801	802	803	804	805	806	807	808	809	810	811	812	813	814	815	816	817	818	819	820	821	822	823	824	825	826	827	828	829	830	831	832	833	834	835	836	837	838	839	840	841	842	843	844	845	846	847	848	849	850	851	852	853	854	855	856	857	858	859	860	861	862	863	864	865	866	867	868	869	870	871	872	873	874	875	876	877	878	879	880	881	882	883	884	885	886	887	888	889	890	891	892	893	894	895	896	897	898	899	900	901	902	903	904	905	906	907	908	909	910	911	912	913	914	915	916	917	918	919	920	921	922	923	924	925	926	927	928	929	930	931	932	933	934	935	936	937	938	939	940	941	942	943	944	945	946	947	948	949	950	951	952	953	954	955	956	957	958	959	960	961	962	963	964	965	966	967	968	969	970	971	972	973	974	975	976	977	978	979	980	981	982	983	984	985	986	987	988	989	990	991	992	993	994	995	996	997	998	999	1000	1001	1002	1003	1004	1005	1006	1007	1008	1009	1010	1011	1012	1013	1014	1015	1016	1017	1018	1019	1020	1021	1022	1023	1024	1025	1026	1027	1028	1029	1030	1031	1032	1033	1034	1035	1036	1037	1038	1039	1040	1041	1042	1043	1044	1045	1046	1047	1048	1049	1050	1051	1052	1053	1054	1055	1056	1057	1058	1059	1060	1061	1062	1063	1064	1065	1066	1067	1068	1069	1070	1071	1072	1073	1074	1075	1076	1077	1078	1079	1080	1081	1082	1083	1084	1085	1086	1087	1088	1089	1090	1091	1092	1093	1094	1095	1096	1097	1098	1099	1100	1101	1102	1103	1104	1105	1106	1107	1108	1109	1110	1111	1112	1113	1114	1115	1116	1117	1118	1119	1120	1121	1122	1123	1124	1125	1126	1127	1128	1129	1130	1131	1132	1133	1134	1135	1136	1137	1138	1139	1140	1141	1142	1143	1144	1145	1146	1147	1148	1149	1150	1151	1152	1153	1154	1155	1156	1157	1158	1159	1160	1161	1162	1163	1164	1165	1166	1167	1168	1169	1170	1171	1172	1173	1174	1175	1176	1177	1178	1179	1180	1181	1182	1183	1184	1185	1186	1187	1188	1189	1190	1191	1192	1193	1194	1195	1196	1197	1198	1199	1200	1201	1202	1203	1204	1205	1206	1207	1208	1209	1210	1211	1212	1213	1214	1215	1216	1217	1218	1219	1220	1221	1222	1223	1224	1225	1226	1227	1228	1229	1230	1231	1232	1233	1234	1235	1236	1237	1238	1239	1240	1241	1242	1243	1244	1245	1246	1247	1248	1249	1250	1251	1252	1253	1254	1255	1256	1257	1258	1259	1260	1261	1262	1263	1264	1265	1266	1267	1268	1269	1270	1271	1272	1273	1274	1275	1276	1277	1278	1279	1280	1281	1282	1283	1284	1285	1286	1287	1288	1289	1290	1291	1292	1293	1294	1295	1296	1297	1298	1299	1300	1301	1302	1303	1304	1305	1306	1307	1308	1309	1310	1311	1312	1313	1314	1315	1316	1317	1318	1319	1320	1321	1322	1323	1324	1325	1326	1327	1328	1329	1330	1331	1332	1333	1334	1335	1336	1337	1338	1339	1340	1341	1342	1343	1344	1345	1346	1347	1348	1349	1350	1351	1352	1353	1354	1355	1356	1357	1358	1359	1360	1361	1362	1363	1364	1365	1366	1367	1368	1369	1370	1371	1372	1373	1374	1375	1376	1377	1378	1379	1380	1381	1382	1383	1384	1385	1386	1387	1388	1389	1390	1391	1392	1393	1394	1395	1396	1397	1398	1399	1400	1401	1402	1403	1404	1405	1406	1407	1408	1409	1410	1411	1412	1413	1414	1415	1416	1417	1418	1419	1420	1421	1422	1423	1424	1425	1426	1427	1428	1429	1430	1431	1432	1433	1434	1435	1436	1437	1438	1439	1440	1441	1442	1443	1444	1445	1446	1447	1448	1449	1450	1451	1452	1453	1454	1455	1456	1457	1458	1459	1460	1461	1462	1463	1464	1465	1466	1467	1468	1469	1470	1471	1472	1473	1474	1475	1476	1477	1478	1479	1480	1481	1482	1483	1484	1485	1486	1487	1488	1489	1490	1491	1492

identified and will be referred to as "normal." Both homologues of chromosomes #I and #II were always present in the near-diploid cells, but, in some instances, parts of them were deleted. Both homologues of chromosome #III were also present in most cells. However, chromosome #VIII, the sex chromosome, and chromosome #V were often found with one homologue of the pair missing. Among the rest of the "normal" chromosomes, such as the metacentric chromosomes #VI and #VII, and the subtelocentrics #IX, #X, and #XI, one homologue of the pair was absent in most cells. The extra chromosomes were generally found to be either telocentric or subtelocentric, although metacentric and submetacentric extra chromosomes were also present in smaller numbers.

Among the 100 cells, 34 different chromosome patterns were found (Table 50). Thus, cells having same number of chromosomes may differ in chromosome composition. For example, there were 15 different chromosome patterns for the cells having 23 chromosomes (Table 50). Even cells of the same pattern may differ in minor ways. In Table 51, pattern D, one group of 26 cells had one more extra submetacentric chromosome and the other of 25 cells had one more telocentric fragment (Figures 72 and 73). However there was no difference in the "normal" chromosomes. The other chromosome patterns were found less frequently. Therefore, the predominant chromosome pattern is pattern D with 23 chromosomes.

TABLE 51

Chromosome complement of 85 metaphase cells of Chinese hamster V cell strain (rearranged from Table 50)

Chromosome pattern	Chromosome count	Cell count	Chromosome #I	Chromosome #II	Chromosome #VIII	Chromosome #III	Chromosome #IV	Chromosome #V	Chromosome #VI	Chromosome #VII	Chromosome #IX	Chromosome #X	Chromosome #XI	Extra chromosomes		
														Metacentric	Submetacentric	Telocentric subtelocentric
A	20	2	2	2	1	2	1	1	1	1	1	1	1	1	3	2
	21	1	2	2	1	2	1	1	1	1	1	1	1	1	2	5
	22	2	2	2	1	2	1	1	1	1	1	1	1	1	1	7
	23	1	2	2	1	2	1	1	1	1	1	1	1	1	1	8
	24	1	2	2	1	2	1	1	1	1	1	1	1	1	3	7
B	21	1	2	2	1	2	1	1	2	1	1	2	1	1	3	2
	22	4	2	2	1	2	1	1	2	1	1	2	1	1	1	4
C	23	4	2	2	1	2	1	1	1	1	2	1	1	1	3	5
	24	1	2	2	1	2	1	1	1	1	2	1	1	1	2	7
D	23	25	2	2	1	2	1	1	1	1	1	2	1	1	1	7
	24	1	2	2	1	2	1	1	1	1	1	2	1	1	1	8
	23	26	2	2	1	2	1	1	1	1	1	2	1	1	2	6
	24	3	2	2	1	2	1	1	1	1	1	2	1	1	2	7
E	22	4	2	2	1	2	1	1	2	1	2	1	1	1	2	4
	23	2	2	2	1	2	1	1	2	1	2	1	1	1	2	4
	24	2	2	2	1	2	1	1	2	1	2	1	1	1	2	6
F	21	1	2	2	1	1	2	1	1	1	1	2	1	1	2	4
	22	1	2	2	1	1	2	1	1	1	1	2	1	1	2	5
G	23	1	2	2	2	2	1	1	2	1	2	2	1	1	1	4
	24	1	2	2	2	2	1	1	2	1	2	2	1	1	1	5

Cells differing in number of chromosomes often had similar patterns of "normal" chromosomes (Figures 65-80). Among 85 cells, we

have recognized 7 different basic chromosome patterns: A (Figures 65-69); B (Figures 70 and 71); C; D (Figures 72-75); E (Figures 76-78); F (Figures 79 and 80); and G, as shown in Table 51. The only difference between cells of any one pattern was in the number of extra chromosomes they possessed. If one or two more chromosomal breaks had occurred in one of the two homologues of chromosomes #VI, #IX, or #X in certain cells of patterns B, C, D, and E, they would have been the same as pattern A. This may also be possible for most of the cells listed in Table 50, with the exceptions of those with patterns F and G. These latter patterns might have been alterations of a more common pattern, caused by the technique of preparation.

Chromosome aberrations

Chromatid breaks and secondary constriction were sometimes found in the large chromosomes. There was no evidence to suggest that the small chromosomes #V, #VI, #VII, and subtelocentrics #IX, #X and #XI had not had aberrations. Since these chromosomes were smaller, the loss of a small fragment, e. g., a telomere, would not have been observed. The loss of one or more chromosomes and the appearance of the extra chromosome were probably the earliest and most frequent aberrations encountered in the cell strain. Deletion was identified only in homologous chromosomes that were of unequal length. In Figure 80, for example, two chromosomes #I had long arms of different lengths. Thus a chromosomal break must have occurred initially, and as a result a telomere was deleted. There are no data concerning the percentage of chromosome breakage available in this study.

Endoreduplication (Figure 81) and chromatid exchanges (Figure 82) were observed in a few cells. The latter were only found in the tetraploid cells.

Discussion

All cells of this line contained several extra chromosomes, which were apparently the broken or rearranged pieces of normal chromosomes once present in the cells. However, it was impossible to recognize from which normal chromosomes the extra chromosomes were derived.

Although classical diploid cells have been reported in this strain by other investigators,^(1,6) we have not found any. This discrepancy may arise from the difficulty in identifying chromosomes after colchicine treatment. In the present studies, the distinctions between chromosomes #III and #IV; #V, #VI, and #VII; and #IX, #X, and #XI are very marked. However, if the short arms of the extra chromosomes, for example, the submetacentrics, become more contracted, they may be confused with one of the normal chromosomes, for example #IX, #X, or #XI, or even with #V,

Figure 81. Endoreduplication in a metaphase cell

Figure 82. Chromosomal exchanges

#VI, or #VII. Ford and Yerganian⁽¹⁾ observed three chromosomes of #VI or #VII, and also three of #X or #XI, in the cells of several strains. Again, this result may be due to chromosome contraction with colchicine. The extra metacentric and submetacentric chromosomes found in this study had arm ratios of 1:2.2. The normal chromosomes #IX, #X, #XI had arm ratios of 1:4.4. It is obvious that the extra chromosomes cannot be confused with these normal chromosomes. However, if certain deletions occur, the difficulty of identification may still exist.

The presence of acentric chromosomes or chromatid fragments resulting from non-centromeric breaks will initially modify the chromosome complement of the cell. Such fragments will be eliminated after one or two cell divisions, and although the chromosome morphology will be abnormal, the chromosome number of this cell will still appear normal. Cells of this strain having chromosome complements of 21 or less, unless chromosomes have been lost accidentally due to preparation, may result from two chromosomal breaks occurring on both arms of one chromosome very close to the centromere, yielding two acentrics and a centromeric fragment, which are eventually excluded. If a break occurs on the centromere of a chromosome two telocentric fragments may be formed. The total chromosome complement of such a cell would be 23. Ford et al.⁽⁶⁾ found that the appearance of two telocentric chromosomes often coincided with the disappearance of a chromosome #III or #VIII, and the lengths of the telocentrics were equal to the arm lengths of the absent chromosome. This finding probably indicated that a break had occurred on the centromere of a chromosome to yield two viable telocentrics. In the present studies, cells with 23 chromosomes have existed for several years. Thus each chromosome must have had a centromere, and must have been able to reproduce. The extra chromosome could have arisen from a centromeric break of one of the initial chromosomes.

Hsu and Somers⁽⁷⁾ used 5-bromodeoxyuridine (BUDR) to induce constrictions and to break chromosomes in Chinese hamster cells. They found that most (86%) of the breaks induced by this compound were at one place, the secondary constriction of chromosome #1. This finding indicates that a specific region of the chromosome is probably most sensitive to damage by environmental factors.

The environmental factors and cytological mechanisms producing aberrations in vitro have been discussed extensively by Chu.⁽⁸⁾ In addition to those he mentioned, metabolic wastes seem also to act as mutagenic agents.⁽⁹⁾ It is suggested here that if the cells are exposed to a given in vitro environment certain aberrations will be induced. If all the possible and still viable abnormalities have been produced, and the cells thereafter are adapted to the in vitro environment, there seem to be no further chromosome aberrations. The most frequently observed cells, those having 23 chromosomes of pattern D, have existed for several years, and there seems to be no reason why such cells could not be maintained in successive subcultures indefinitely.

Summary

After several years of transfer and subculture in different laboratories with different media and sera, the Chinese hamster V cell strain still contains the same percentage near-diploid cells. The majority of the cells contain 23 chromosomes, and most of these possess the same chromosome pattern, which is, therefore, taken as representative of the line. However, 34 different types of chromosome constitution were found in the cells studied. With some exceptions the cells having different complements are nevertheless similar in chromosome constitution. On the other hand, cells having the same number of chromosomes may be of different patterns. The aberrations which give rise to changes in chromosome morphology and number seem to occur only on certain chromosomes and in certain regions of these chromosomes. Hence, the difference between cells having different chromosome complements depends upon the number of extra chromosomes that have appeared. The "normal" chromosomes of the same type present in cells of different chromosome complements are very similar.

References

1. Ford, D. K. and G. Yerganian. Observations on the chromosomes of Chinese hamster cells in tissue culture. *J. Natl. Cancer Inst.* 21, 393-425 (1958).
2. Greenblatt, C. L. The evaluation of X-ray induced chromosome aberrations in cell cultures of the Chinese hamster. *Intern. J. Rad. Biol.* 4 (2), 185-210 (1961).
3. Sinclair, W. K. X-ray-induced heritable damage (small colony formation) in mammalian cells in culture. (To be published).
4. Elkind, M. M. and H. Sutton. X-ray damage and recovery in mammalian cells in culture. *Nature* 184, 1293-1295 (1959).
5. Hsu, T. C. and Klatt, O. Mammalian chromosomes in vitro. IX. On genetic polymorphism in cell populations. *J. Natl. Cancer Inst.* 21, 437-474 (1958).
6. Ford, D. K., R. Wakonig, and G. Yerganian. Further observations on the chromosomes of Chinese hamster cells in tissue culture. *J. Natl. Cancer Inst.* 22, 765-799 (1959).
7. Hsu, T. C. and C. E. Somers. Effect of 5-bromodeoxyuridine on mammalian chromosomes. *Proc. Natl. Acad. Sci. U.S.* 47, 396-403 (1961).

8. Chu, E. H. Y. Chromosomal stabilization of cell strains. Syverton Memorial Symposium: Analytic cell culture. Natl. Cancer Inst. Monograph 7, 55-74 (1962).
9. Haas, F. L. and C. O. Doudney. A relation of nucleic acid synthesis to radiation-induced mutation frequency in bacteria. Proc. Natl. Acad. Sci. U.S. 43, 871-883 (1957).

PRELIMINARY EXPERIMENTS WITH THE "SPONGE TECHNIQUE"
FOR ELECTRON MICROSCOPIC STUDIES OF FIBRILLOGENESIS
IN THE REGENERATING TENDON

F. Wassermann

Electron microscopic studies on the formation of collagenous fibers by several investigators using various materials have shown that in the beginning of fibrillogenesis fine fibrillar structures of $< 100 \text{ \AA}$ appear within the fibroblasts, accumulating in a cortical or exoplasmic zone of the cell.⁽¹⁻⁷⁾ The question arose whether these "primary fibrils"⁽¹⁾ are the precursors of the cross-banded collagenous fibrils, which, in more advanced fibrillogenesis, are found at the surface of the fibroblasts. Although some investigators are inclined to answer this question in the affirmative, all agree that evidence for a direct relationship between primary and collagenous fibrils is still lacking. If the intracellular fibrous structures were the precursors of the extracellular fibrils, one would expect the fibroblast to discharge the primary fibrils by a kind of apocrine secretion, in which case the cell membrane would be lost temporarily during this (probably very short) period. Exhaustive investigation of this critical phase is required in order to decide whether the fibroblast undergoes the profound surface changes involved in the discharge of fibrillar material and, possibly, of the entire exoplasmic zone, or whether, as some investigators maintain,⁽⁸⁾ only monomeric precollagenous material is released by the cell, with subsequent formation of collagenous fibrils by extracellular polymerization.

Since previous investigations have not resulted in an answer to this question, new ways of modifying the activity of the fibroblast at the beginning of fibrillogenesis have been sought. The possibility of increasing the frequency of occurrence of the critical phase by delaying or arresting the cellular processes involved appeared promising.

A method which seemed to serve our purpose by opening new ways of studying fibrillogenesis was found in what we may call the sponge technique. This consists of the implantation of a biologically inert or nontoxic porous material into the gap between the stumps of a transversely cut Achilles tendon in rats. It has been shown with the light microscope that the interstices of such sponges become gradually filled with young connective tissue produced by fibroblasts that move into the sponge. The method has not yet been applied to detailed histological, histochemical and electron microscopic studies.

Previous investigators⁽⁹⁾ have used the Ivalon Surgical Sponge (Clay-Adams Co., Inc.; New York), which was inserted into experimental wounds. This plastic material is inert and is easy to implant and remove.

Another material that also seemed suitable for our purpose and that has certain advantages over the plastic sponge is the absorbable gelatin sponge "Gelfoam" (Upjohn Co.; Kalamazoo, Mich.). This porous material also becomes invaded by connective tissue cells and is gradually replaced by connective tissue. Since we are interested in the early stages of fibrillogenesis, which occur about the fourth to the sixth day after implantation of the sponge, the slow absorption of the gelatin should not interfere with our experiments. The present preliminary report is concerned with the Gelfoam sponge experiments.

Experimental procedure

Tenotomy of the Achilles tendon was performed in rats of 300 to 500 grams body weight, as described in an earlier paper.⁽¹⁾ The sponge was prepared by cutting small cubes, about 2 mm on a side, from pieces of Gelfoam sterilized in the autoclave at 250°C for four hours. The sponges were put in sterile salt solution and were inserted into the wound immediately after tenotomy. The wound was closed over the sponge by a single stitch and covered with penicillin powder. Animals were sacrificed at intervals from three to eleven days after the operation. For light microscopy the entire wound region was fixed in Zenker's fluid plus 10 per cent formalin; for electron microscopy the sponge was excised and quickly divided into pieces of about 1 mm in diameter that were fixed in 1 per cent buffered osmic acid (pH 7.4) on ice for an hour. After dehydration in ethanol the tissue samples were embedded in Epon. Sectioning was done with the Porter-Blum microtome. Photographs were taken with the RCA EMU 3F electron microscope.

Observations

Figure 83 shows the gelatin sponge in the center of the wound region seven days after the operation. Only the proximal stump of the severed tendon is seen in this section. The sponge is surrounded by newly formed connective tissue.

Figure 84 presents a peripheral area of the same sponge seen in Figure 83. In the meshes of the sponge several isolated fibroblasts are seen, apparently moving in from the surrounding tissue. No fibrous structures are present in the vicinity of the fibroblasts.

Figure 83

Longitudinal section of the area of the severed Achilles tendon showing the young connective tissue between the stumps of the tendon. Only the proximal stump is seen in this section. The arrow points to the sponge material. Notice that seven days after the implantation of the sponge no foreign body reaction or inflammation has developed. Fixation Zenker-formalin solution; hematoxylin-eosin staining.

Figure 84

Part of the periphery of the sponge showing isolated fibroblasts (arrow) moving into the interstices of the sponge.

The electron microscopic aspect of fibroblasts under the same conditions as those in Figure 84 is presented in Figure 85. The characteristic fine structure of the fibroblast, i.e., the richly developed endoplasmic reticulum with rather wide lacunae, rosettes of ribosomes in the groundplasm, and a nucleus with rather evenly distributed chromatin material, is evident. The portion of a fibroblast, seen at higher magnification in Figure 86, shows in addition a considerable amount of Golgi material. It is of interest to compare the fibroblast with the typical macrophage (Figure 87), which, apart from the fibroblast, is the cell type most commonly seen in the sponge. This cell differs from the fibroblast by the denser nucleus, much less conspicuous endoplasmic reticulum, greater number of smaller mitochondria, and by the finger-like pseudopodia emerging from its surface.

Figure 85. Isolated fibroblast, seven days after implantation of the sponge, attached to gelatin strand of the sponge. Notice the characteristic endoplasmic reticulum (en), the structure of the nucleus (n) and the cytoplasm, especially the exoplasmic zone (ez) where fine fibrillar structures are present.

Figure 86. Part of fibroblast, 8 days after implantation of the sponge. Collagenous fibrils (cf) have already formed. At the left is the exoplasmic zone (ez) with fibrous structures. The surface of the cell in this area is fuzzy. n, nucleus, en, endoplasmic reticulum, m, mitochondrion, gm, Golgi material.

Figure 87. One of the commonly seen macrophages in the sponge. The cell seems to engulf an extravasal erythrocyte. Notice how the macrophage differs from the fibrocyte in the structure of the nucleus (n), in the endoplasmic reticulum (en), and in the mitochondria (m).

The special feature of the fibroblast in Figure 85 is the distinct cortical zone, free of endoplasmic reticulum, ribosomes and mitochondria, which is characteristic of the very beginning of fibrillogenesis. The fine fibrous structures in the exoplasm (whose relationship to the collagenous fibrils is still uncertain) can be seen even at the relatively low magnification of this figure. The fibroblast taken at the eighth day after implantation (Figure 86) displays a more advanced stage of fibrillogenesis than that in Figure 85. At the left one notices the fuzzy character of the cell surface, which seems to us characteristic of the critical phase of fibrillogenesis and suggests the discharge of exoplasmic material by the cell. Some collagenous fibrils have already been formed near the surface of the cell (top, at the right).

Conclusions

Our preliminary experiments have shown that the gelatin sponge technique is a very useful procedure for obtaining early stages of fibrillogenesis in isolated fibroblasts in vivo as material for electron microscopic studies. The experiments will be continued. The intention is to influence the cellular processes involved in fiber formation by local application of chemical and other agents with which the gelatin sponge can be imbibed. Experiments with the plastic sponge have also been started. The plastic sponge will be especially useful for studying systemic influences on the isolated cells. In addition to serving our immediate purpose, this method of isolating cells within the tissue in vivo is expected to open new opportunities for electron microscopic cell studies, among them studies of the effects of irradiation on such cells.

References

1. Wassermann, F. Fibrillogenesis in the regenerating rat tendon with special reference to growth and composition of the collagen fibril. *Am. J. Anat.* 94, 399-438 (1954).
2. Wassermann, F. Electron microscopic study of fibrillogenesis in the regenerating tendon of the guinea pig controlled by vitamin C-deficiency and readministration. *Anat. Rec.* 130, 386 (1958).
3. Jackson, S. F. The morphogenesis of avian tendon. *Proc. Roy. Soc. (London) B* 144, 556 (1956).
4. Porter, K. R. and G. T. Pappas. Collagen formation by fibroblasts of the chick embryo dermis. *J. Biophys. Biochem. Cytol.* 5, 153-166 (1959).

5. Godman, G. C. and K. R. Porter. Chondrogenesis studied with the electron microscope. J. Biophys. Biochem. Cytol. 8, 719-760 (1960).
6. Giesecking, R. Elektronoptische Beobachtungen an Fibroblasten. Struktur und Stoffwechsel des Bindegewebes. II. Symposium an der mediz. Klinik Muenster. Georg Thieme Verlag, Stuttgart, 1960.
7. Ross, R. and E. P. Benditt. Wound healing and collagen formation. J. Biophys. Biochem. Cytol. 11, 677-700 (1961).
8. Karrer, H. E. The fine structure of connective tissue in the tunica propria of bronchioles. J. Ultrastructure Res. 2, 96-121 (1959).
9. Boucek, R. J. and N. L. Noble. Connective tissue, a technique for its isolation and study. A. M. A. Arch. Pathol. 59, 553-558 (1955).

PRELIMINARY EXPERIMENTS ON LABELING OF GRASSHOPPER SPERMATOGONIA WITH TRITIATED THYMIDINE

Theodore N. Tahmisian and Rosemarie L. Devine

The testis of the grasshopper provides excellent material with which to study cellular changes that occur during spermatogenesis. The effect of X-irradiation on organelle differentiation, and its consequent value in the interpretation of normal processes, have already been reported.⁽¹⁾ To augment our study of the earlier stages of spermatogenesis, we have now undertaken experiments with tritiated thymidine. Results are still preliminary.

The tritium-labeled material should provide considerable information about the relationship of the nucleus or the chromosomes to the differentiation processes. Distribution and intensity of grains will determine whether thymidine can be incorporated in several generations of developing spermatogonia, or whether it is incorporated in only one developmental stage. It has been noted in mammals that uptake of thymidine occurs in both the spermatogonia and preleptotene spermatocytes.⁽²⁾ We are interested in finding whether this is also true in grasshoppers.

The H^3 -thymidine, in distilled water, was prepared by W. Kisielewski of this Division. A pilot experiment was conducted to devise a method of injecting the grasshoppers with small amounts of H^3 -thymidine, and then to determine practical dosage, sacrifice times and exposures of autoradiographs. Injections were made from a 1/4-cc syringe, fitted with a 30-gauge needle and controlled by an attached micrometer, into the abdominal cavity of the insects. It was found that the grasshoppers could not survive $> 10 \mu c$ of the thymidine. However, injections of $5 \mu c$ ($1 \mu c/\mu l$) were tolerated. It was also found that the labeling occurred randomly in several spermatogenic cysts of a testicular follicle. This implies either variation in the rate of descent of the developing cysts down the follicle or uptake of the thymidine for an unexpectedly long period of time after injection. To clarify this, more frequent sacrifices are being made. The testes are removed and prepared as for paraffin sections, cut at 3 microns, mounted on glass slides, and prepared for autoradiography according to the (unpublished) method of S. Leshner of this Division. Exposure time is generally two weeks.

Preliminary experiments include autoradiographic studies to establish: 1) the period or periods of uptake of the isotope, and 2) whether grasshopper spermatogenesis is cyclic, as in mammals. Also, some treated material has been prepared for the electron microscope to look for possible deleterious effects of the H^3 -thymidine.

References

1. Tahmisian, T. N. and R. L. Devine. The influence of X-rays on organelle induction and differentiation in grasshopper spermatogenesis. J. Biophys. Biochem. Cytol. 9, 29-45 (1961).
2. Nebel, B. R., C. J. Murphy and H. J. Linder. Autoradiographic study with tritiated thymidine of mouse testis after 320 r and after 1000 r of acute localized X-irradiation. Radiation Res. 13, 126-136 (1960).

AN EXAMINATION OF EMBRYOLOGICAL AND MYOGENETIC
DEVELOPMENT IN THE ZEBRAFISH, BRACHYDANIO RERIO
(HAMILTON-BUCHANAN)

Theodore N. Tahmisian, Betty Jean Wright
and Bronson R. Hall

In order to extend the analysis of muscle differentiation, which was begun by a study of the grasshopper embryo, Melanoplus differentialis differentialis,⁽¹⁾ a study of myogenesis in another organism was initiated. Glücksmann⁽²⁾ reported that the origin of striated muscle and its mode of differentiation may vary in different species and possibly even in the muscles of the same species. Since examination of the formation of grasshopper muscle has yielded findings different from those described in the classical work of Heidenhain,⁽³⁾ who used trout embryos, it seemed appropriate to investigate this phenomenon in an organism closer to that used by Heidenhain, namely the embryo of the zebrafish, Brachydanio rerio.

The zebrafish was chosen for several reasons: 1) it may be bred conveniently under laboratory conditions with a number of eggs obtained at one time; 2) the time required from fertilization of the egg to hatching is only 60 hours (at $26 \pm 1^\circ\text{C}$); 3) gross development of the embryo may be followed fairly easily because of the extreme transparency of the chorion; 4) the cell nuclei may be easily observed in the living state with the phase contrast microscope; 5) the cells are of convenient size for electron microscopy.

This report presents the results of the study of the developmental stages of B. rerio together with a light microscope study of myogenetic sequences in formation of striated muscle in the embryo. The ultimate purpose of this study is to correlate, if possible, the myogenetic sequences in the fish with those in the grasshopper.

Although several different breeding methods for the zebrafish have been suggested,⁽⁴⁻⁸⁾ we have chosen to use a five-gallon battery jar with a layer of 0.5 cm glass beads placed on the bottom and water added to a level of about 5.0 cm. The glass beads and limited amount of water prevent the fish from eating their own eggs as they are being deposited. Fish that are ready to spawn are placed in the breeding jar described above in the ratio of at least two males to one female. Live food, such as Daphnia, Tubifex, brine shrimp and white worms (Enchytrae), is supplied in abundance just previous to spawning. Breeding activity is usually observed within an hour after the fish are put together, if the fish are ready to spawn. It was found advantageous to keep the male and female breeders separated in the aquarium to prevent the females from laying eggs before they were needed. Each spawning act releases 5 to 10 eggs, and one female may deposit as many as 100 eggs in 30 minutes.

Zebrafish eggs are approximately 0.5 mm in diameter, transparent, and easily visible to the naked eye. Dead eggs, recognizable by their opacity and whiteness in reflected light, could be discarded when the eggs were collected with a pipette from the breeding container. The eggs were put into "aged" water in 7.0-cm crystallizing dishes and kept in a constant temperature water bath at $26 \pm 1^\circ\text{C}$. The living material was photographed at specific times of development and was subsequently fixed in Bouin's fluid. The fixed material was then embedded in paraffin, sectioned, stained and counter-stained. The three stains used were. Heidenhain's iron hematoxylin, Mayer's acidified hematoxylin, and Schiff's reagent for the Feulgen staining reaction. Eosin, Fast Green and Orange G were employed as counter-stains. An examination of the photographs of the living embryos, together with written descriptions and sketches made during development, made it possible to divide the developmental period into a number of stages extending over the 60-hour interval. The slides prepared from the fixed material were used to study the myogenetic processes of striated muscle tissue.

The developmental stages of the zebrafish have been worked out by Hisaoka and Battle (9) In our hands, however, development is much more rapid; the embryos are recognizable as baby fish within the first 24 hours, and they hatch during the third day, contrary to the 96-hour hatching time given by Hisaoka and Battle.(9) The 60-hour developmental period can be conveniently divided into 24 stages that are characterized by gross morphological changes in the embryo observed in the living state with the aid of a dissecting microscope.

The egg is fertilized externally immediately upon being deposited by the female (Stage 1). Twenty-five minutes after fertilization the first cleavage occurs, giving a two-celled ovum. Stages 2 through 8 (the two-celled ovum through the 64-celled ovum) take approximately two hours, or about 20 minutes per cleavage. Blastulation gives rise to a flattened disc-like structure overlying the deutoplasm. Blastulation can be divided into four discernible stages (Stages 9 through 12). the early high blastula, the late high blastula, the flat blastula and the very late blastula. Gastrulation consists of the expansion of the blastoderm over the yolk and the formation of the germ ring and the embryonic shield. Gastrulation can be divided into three stages (Stages 13 through 15): the early gastrula, the middle gastrula with the blastoderm covering one-half of the yolk sphere, and the late gastrula with the blastoderm covering three-fourths of the yolk sphere. The processes of blastulation and gastrulation take approximately six hours to complete. About nine hours after fertilization the blastopore is closed (Stage 16), and the embryo is detectable as a translucent ridge almost encircling the yolk sphere. The first of the mesodermal somites have become differentiated during this stage and the somites continue to form during the ensuing stages, until a full complement of 34 somites is present at Stage 23. This cephalo-caudal sequence of differentiation of the somites provides areas of striated muscle development in different stages in one embryo.

Thirteen hours after fertilization the first visible differentiation of the head region of the embryo has occurred with the outpouching of the fore-brain walls to form the optic vesicles (Stage 17). Stage 18 (15 hours after fertilization) is characterized by the invagination of the ectoderm to form the auditory placodes. After 19 hours of development (Stage 19) the optic vesicles invaginate to form the optic cup, and the lens begins to develop from the overlying ectoderm. This stage also shows the elevation of the tail from the yolk mass because of the increased length of the embryo. About 21 hours after fertilization (Stage 20) there is further differentiation of the auditory placode when the otoliths are formed. It is at this time that the first indications of striated muscle contractions are observed, there are slight twitchings of the embryo that start in the most cephalad somites. This movement increases and extends more caudally during development. The heart appears on the anterior surface of the yolk sac as a flattened tube and begins to pulsate irregularly, although no cardiac muscle striations can be seen at this time or even at later times closer to hatching. By 24 hours after fertilization (Stage 21) the retinal layer of the eye becomes pigmented and the head region is free of the yolk. After the appearance of melanin in the retina, melanophores become massed over the head and yolk sac, along the dorso-lateral musculature, and ventrally over the region of the intestine and ventral musculature. This extensive body pigmentation (Stage 22) begins approximately 30 hours after fertilization and continues even after hatching until the characteristic pattern of longitudinal stripes, which gives the zebrafish its name, appears. Prehatching Stage 23, at 50 hours after fertilization, marks the appearance of the xanthophores, yellow pigment cells, in the region of the eyes and the general head area, which ultimately spread over the whole body surface. The embryo if removed from the chorion, now resembles a newly hatched larval fish and is capable of survival. Hatching (Stage 24), which occurs approximately 60 hours after fertilization, is achieved by the lashing movements of the tail that eventually rupture the egg capsule and release the larval fish. Even under controlled, uniform temperature conditions there is considerable variation in hatching time. It has been suggested by Blumenkrantz⁽¹⁰⁾ that this variation in hatching time is due to the physical forces acting on the inanimate chorion, rather than developmental forces applied to a living membrane.

The striated muscle tissue of the teleosts arises from the mesoderm and in particular from the somitic myotomes.⁽¹¹⁾ Cells giving rise to muscle tissue are called myoblasts. They are located within the myotome and are at first regular and cylindrical, but in time they become spindle-shaped and are arranged in parallel bundles. During this time they multiply rapidly by mitosis, myofibrils then appear within the myoblasts, and finally the characteristic cross striations develop.

Histological studies of tissue differentiation and, more particularly, myogenetic processes in teleosts are relatively rare.⁽¹²⁾ In an examination

of the fixed material of the zebrafish it was possible to find the developmental stages of striated muscle as classified by Tello.^(13,14) The following stages are to be identified: 1) the myoblast, in which no distinction can be made between the potential muscle cells and other closely associated fibroblasts; 2) the myocyte, in which the uni- or multi-nucleated cell has become elongated, the cytoplasm retaining its homogeneity; 3) the myotube, in which the elongated cell begins to reveal striation at its periphery; 4) the muscle fiber, in which transverse striation has become fully established and the nuclei are situated at the periphery. Boyd⁽¹²⁾ has suggested that better terms for Tello's Stages 1 and 2 would be the premyoblast and myoblast, respectively. As these terms seem more accurately descriptive of the myogenetic process, they will be used in the further discussion.

Study of the sectioned embryos revealed that the beginning of myogenesis can be found as early as Stage 16 (9 hours after fertilization), when the first somites appear. Because of the cephalo-caudal sequence of differentiation of the somites it is possible to find examples of all of the stages in the myogenetic process in various somites of the same embryo. The best material for analysis of myogenesis in the zebrafish, however, comes from embryos 21 hours of age (Stage 20) or older, by which time muscle contractions can be seen.

The premyoblasts are represented by elongated cells in areas of mitotic activity. These cells can be distinguished from other fibroblasts of mesodermal origin only by their position in relation to the subsequent developmental history of the region. Myoblasts (Figure 88) can be identified, if in a region of myogenesis, by their large size and elongated shape and by the small number of mitotic figures in the field. The cytoplasm of the myoblasts increases in volume and takes on a granular and vesicular aspect. As development continues the myoblast elongates still further and becomes multi-nucleated. The nuclei align themselves centrally and the myofibrils begin to appear between the nuclei (Figure 89). The cytoplasm becomes clearer. The myofibrils are at first homogeneous and few in number. Usually one fibril becomes striated before the others and begins to divide longitudinally with the two ends sometimes remaining homogeneous or non-striated (Figure 90). During myofibrillar differentiation the nuclei of the myoblasts tend to crowd together centrally, retaining their oval shape. This structural change results in the myotube stage (Figure 90). The nuclei then migrate to the margin of the myotube and the myofibrils occupy the central position of the muscle fiber (Figure 91) to complete the process.

Embryonic myoblasts and regenerating striated muscle are generally known to be endowed with an abundance of nuclei; most of the myoblastic body is nuclear rather than cytoplasmic. The resultant differentiated striated muscle, however, contains comparatively few nuclei and

these are restricted to the periphery of the fibril bundles.⁽¹⁵⁾ The fate of the other nuclei has never been clearly described.⁽¹²⁾ When Figures 88 and 91 are compared, loss of nuclear material, similar to that found in the grasshopper, seems to occur also in the zebrafish.

Figure 88. Section of an embryo (Stage 20) in the region of a mesodermal somite (S) showing the myoblasts with their nuclei (N) and the granular and vesicular cytoplasm (C). The notochord (NC) lies adjacent to the mesodermal tissue. Bouin's fixation; Mayer's hematoxylin and eosin.

Figure 89. Section of an embryo (Stage 21) showing the alignment of the nuclei (N) with condensed chromatin and the cytoplasm (C) which is less granular than in earlier stages. Close observation reveals the beginnings of myofibril formation between the nuclei. Bouin's fixation; Mayer's hematoxylin and eosin.

Figure 90. Section of an embryo (Stage 22) showing the myotube (MT) with the oval nuclei centrally aligned. The cytoplasm has cleared. A dividing myofibril (DM) shows the longitudinal splitting and the presence of striation throughout, except at the ends which are homogeneous. Bouin's fixation; Heidenhain's iron hematoxylin and eosin.

Figure 91. Section of an embryo (Stage 23) showing fully striated muscle fibers. There is a marked decrease in the number of nuclei. Regions of condensed chromatin represent the remaining nuclei. Bouin's fixation; Heidenhain's iron hematoxylin and eosin.

Further examination of the sectioned material reveals a general scattering of basophilic bodies (Figure 92) among the developing myofibrils

Figure 92. Section of an embryo (Stage 22) showing a general scattering of basophilic bodies (B) among the differentiating myofibrils. These bodies are indistinguishable from the condensed chromatin within the intact nuclei (N). Bouin's fixation; Heidenhain's iron hematoxylin and eosin.

and closely related to them. These bodies are indistinguishable in appearance from the condensed chromatin material located within the nuclear envelope. However, staining with the Schiff reagent in the Feulgen reaction indicated that the DNA remains within the nucleus during myogenesis. Since the limits of resolution of the light microscope prevent visualization of the source and fate of the basophilic bodies, further study with the electron microscope and additional cytochemical tests are needed.

References

1. Tahmisian, T. N. An electron microscope study of sarcoblast karyokinesis during myogenesis in grasshopper embryos. *Acta Anat.*, in press.
2. Glücksmann, A. Über die Entwicklung der quergestreiften muskulatur und ihre funktionellen Beziehungen zum Skelet in der Onto- und Phylogenie der Wirbeltiere. *Ztschr. Anat. Entwicklungs.*, 103, 303-370 (1934).
3. Heidenhain, M. *Plasma und Zelle*, G. Fischer, Jena, 1911.
4. Creaser, C. W. The technic of handling the Zebra fish (Brachydanio rerio) for the production of eggs which are favorable for embryological research and are available at any specific time throughout the year. *Copeia* 4, 159-161 (1934).
5. Roosen-Runge, E. On the early development - bipolar differentiation and cleavage - of the Zebra fish, Brachydanio rerio. *Biol. Bull.* 75, 119-133 (1938).
6. Roosen-Runge, E. Karyokinesis during cleavage of the Zebra fish Brachydanio rerio. *Biol. Bull.* 77, 79-91 (1939).
7. Battle, H. I., and K. K. Hisaoka. Effects of ethyl carbamate (urethan) on the early development of the teleost Brachydanio rerio. *Cancer Res.* 12, 334-340 (1952).
8. Innes, W. T. *Exotic Aquarium Fishes*. Innes Publishing Co., Philadelphia, 1953. p. 208.
9. Hisaoka, K. K., and H. I. Battle. The normal developmental stages of the Zebrafish, Brachydanio rerio (Hamilton-Buchanan). *J. Morphol.* 102, 311-327 (1958).

10. Blumenkrantz, B. L. The chronological development of the Zebra fish Brachydanio rerio (Hamilton) at a constant temperature. Unpublished Master of Science thesis, Oklahoma Agricultural and Mechanical College, 1954.
11. Witschi, E. Development of the Vertebrates. W. B. Saunders Company, Philadelphia, 1956. pp. 212-222.
12. Boyd, J. D. Development of striated muscle. Structure and Function of Muscle, ed. G. H. Bourne. Academic Press, New York, 1960. pp. 63-85.
13. Tello, J. F. Trabajos lab. invest. biol. univ. Madrid, 15, 101 (1917). Cited by J. D. Boyd, Reference 12.
14. Tello, J. F. 1922. Ztschr. Anat. Entwicklungs., 64, 348 (1922). Cited by J. D. Boyd, Reference 12.
15. Maximow, A. A., and W. Bloom. A Textbook of Histology. W. B. Saunders Company, Philadelphia, 1961. p. 176.

METABOLIC BASES OF THE IMPAIRMENT OF ROOT INITIATION BY IONIZING RADIATION

Solon A. Gordon, Lowell Woodstock and Bhagwan K. Gaur

This report is a continuation of preliminary studies on the effect of gamma irradiation on the physiology of rooting of excised *Coleus* leaves. When leaves are excised and their petioles placed in water, adventitious roots are formed on the petiole bases within 5-6 days after excision. The number of roots increases with time. The formation of such roots was delayed by exposure of the lamina to X- or gamma radiation, the degree of inhibition being roughly proportional to the dose. The inhibition of rooting by 15 kr of gamma radiation is shown in Table 52. This dose level was used throughout the experiments to be reported here.

TABLE 52

Effect of irradiation of the lamina of excised *Coleus* leaves on root formation

Experiment	Treatment	Days after irradiation				
		6	7	8	9	10
		Roots per petiole*				
1	Control	-	6.7	7.7	9.0	10.0
	Irradiated, 15 kr	-	2.6	3.2	3.4	4.2
2	Control	6.1	8.3	10.3	11.1	-
	Irradiated, 15 kr	0.8	2.4	3.6	4.4	-
Average: Irrad./Control		0.13	0.33	0.38	0.39	0.42

*Each value is the mean of 12 cuttings.

Total sugar, soluble and residual nitrogen, and "nucleotide" levels in the 0.5-cm petiole bases were determined at various times after irradiation and compared with the controls. No effect of irradiation on the carbohydrate and nitrogen relations was observed. However, the absorbancy at 260 m μ (E_{260}) of 5% perchloric acid extracts was reduced in the irradiated series (Table 53). This effect could be detected as soon as 12 hours after irradiation, and indicated that irradiation might inhibit root initiation via an influence on nucleic acid metabolism. However, the above hot perchloric acid extracts may not be directly equated with nucleic acids since no

preliminary extractions were made to remove interfering substances, and absorption spectra were not obtained. Furthermore, the method did not distinguish between RNA and DNA. Accordingly, we are examining in greater detail the effects of gamma irradiation of the lamina on the nucleic acid metabolism of the petiole bases.

TABLE 53

The effect of irradiation of the lamina of *Coleus* leaves
on the absorbancy of hot perchloric acid extracts
of petiole bases

Hours after irradiation	E ₂₆₀		Irradiated as % of control
	Controls	Irradiated	
0	1.90	1.90	100
12	1.76	1.66	94
24	1.96	1.73	88
48	2.27	1.90	84
72	3.17	2.46	78

A single clone of *Coleus blumeii* (variegated) was grown from stem cuttings at 21°C, 60-70% relative humidity, under 16-hour day length. The light intensity was ca. 2000 f.c. from a mixed fluorescent tube-tungsten filament panel at a wattage ratio of 5 to 1. Opposite leaves were excised in pairs from the 3rd to the 5th nodes of vegetative stems and placed in beakers with their petioles immersed in water. One member of each pair was irradiated, the other was the paired control. Leaves were irradiated in the high-level gamma room in batches of 10 or 20, with the petioles shielded by lead brick. The 6000-curie Co⁶⁰ source was placed at a distance of 18 inches from the leaves. Exposures lasted one hour, the total dose being 15 kr. Ambient light intensity was ca. 25 f.c. The control leaves were exposed to similar light intensities outside the gamma room. After irradiation, the leaves were returned to the growth chambers, at a light intensity of 150 f.c., and each petiole was immersed in water in an opaque bottle. Each lamina was supported on a wire loop attached to the mouth of the bottle.

The basal 0.5 cm of the petioles was excised 0, 12, and 36 hr after irradiation, immersed in liquid nitrogen, weighed, and homogenized in ice-cold ethanol. Successive extractions with ethanol, 1:1 ethanol:ether, ethanol, and 0.2 *N* HClO₄ removed phospholipids and acid-soluble nucleotides. The residue was extracted with 0.2 *N* KOH at 37°C for 4 hr and then acidified with an excess of HClO₄. This left a relatively "clean" RNA extract which was decanted, neutralized, and brought to standard volume. Absorption spectra of the RNA extracts were measured between 220 and 310 mμ. The extracts

had well-defined maxima at 258 $m\mu$ and minima at 236 $m\mu$. The $E_{240}:E_{260}$ and $E_{280}:E_{260}$ ratios were constant throughout the experiments, but they were higher than those of the standard yeast RNA preparation. The concentration of RNA in extracts was determined by comparing their $E_{260}-E_{290}$ values with those of the standard.⁽¹⁾

Twelve hours after irradiation, the levels of RNA in the petiole bases were less than in the unirradiated controls (Table 54); the difference was more pronounced at 36 hr. In order to determine whether or not these differences were associated with differences in fresh weight in the petiole bases (bases become noticeably swollen several days before the emergence of the roots), the petiole bases were routinely weighed. No significant differences in fresh weight between irradiated and control bases during the first 36 hr after irradiation were found. It may be inferred that gamma irradiation of the lamina results in a decrease in the RNA content of the petiole bases.

TABLE 54

Effect of irradiation of the lamina on the RNA content of petiole bases. Each datum based on 20 leaves

Hours after irradiation	Treatment	μg RNA/0.5-cm petiole base				
		Exp. #1	Exp. #2	Exp. #3	Exp. #4	Average
0	Control	8.62	8.31	7.24	8.36	8.11
		7.93	8.25	8.10	8.07	
12	Control	9.62	8.94	9.25	9.10	9.24
12	Irradiated	9.96	8.94	9.05	9.02	8.23
		8.45	8.55	8.84	8.01	
		7.97	7.84	8.42	7.77	
36	Control	-	-	10.11	10.17	10.1
36	Irradiated	-	-	10.05	9.87	7.74
		-	-	7.17	8.40	
		-	-	7.15	8.24	

Two-way analysis of variance, I and II

Chance probability between experiments: $>.05$

Chance probability between treatments: $<.001$

The decrease in RNA content in the irradiated series could have resulted from relative inhibition in the rate of RNA synthesis, relative stimulation in RNA degradation, or a combination of these. An attempt was made to distinguish between these possibilities by measuring the uptake of uracil-2- C^{14} into the RNA extracts. Leaves were removed from the culture

jars and their petioles were inserted individually into small vials containing 0.4 ml of uracil-2- C^{14} ($10 \mu\text{C}/\text{ml}$). The lamina were supported at approximately 45° from the vertical. Environmental conditions were maintained as above. After 1-hr incubation with C^{14} -uracil, the petioles were rinsed 3 times with distilled water, and the basal 0.5 cm was excised and extracted for RNA. The solutions remaining in the vials were pooled and measured to give an estimate of the volume absorbed by the plant tissues (evaporation was negligible).

The incorporation of uracil-2- C^{14} into RNA is shown in Table 55. If it is assumed (see below) that total activities in the RNA extracts are proportional to the rates of RNA synthesis during incubation, then it would appear that the rate of RNA synthesis decreases with time and to the same extent in both irradiated and control leaves. It may be concluded that the relative decrease in RNA content in the irradiated series (Table 54) was caused by an increase in the rate of RNA degradation, i.e., in ribonuclease activity. An increase in RNAase activity has been observed in seedlings grown from X-irradiated corn seed.⁽²⁾

TABLE 55

The effect of irradiation on the incorporation of uracil-2- C^{14} into the RNA of petiole bases.
Each datum represents 20 pooled bases.

Hours after irradiation	Treatment	Counts per minute/RNA extract				
		Expt. 1		Expt. 2		Average
		Rep #1	Rep #2	Rep #1	Rep #2	
0	Control	1760	1610	1590	1710	1670
12	Control	1270	1360	1160	1270	1260
12	Irradiated	1220	1380	1230	1170	1250
36	Control	1150	1230	873	867	1030
36	Irradiated	1110	1400	842	941	1070

Although the decreased incorporation of uracil- C^{14} into the RNA extracts indicates a decrease in the rate of RNA synthesis with time, the amounts of RNA per petiole base increased in the controls and remained nearly constant in the irradiated series. These observations suggest that in both series the ribonuclease activity decreased with time, although the extent of the decrease was less in the irradiated series.

Measurements of the uptake of C^{14} -uracil solution by the leaves (Table 56) failed to show any marked differences between treatments, despite the fact that irradiated leaves wilted more easily. Two possible

explanations are that irradiation caused leakage of water into the intracellular spaces of the lamina and thus a loss of turgor, possibly because of damage to the cellular membranes.⁽³⁾ or that irradiation caused a more rapid loss of water from the lamina to its surroundings. It might prove possible to distinguish between these alternatives by determining the fresh weight of irradiated and control leaves at various degrees of wilting. Since increased wilting was the first physiological response observed after irradiation, further investigation of this aspect would seem to be warranted.

TABLE 56

The effect of irradiation on the uptake of uracil-2-C¹⁴ solution by excised Coleus leaves

Hours after irradiation	Treatment	ml/hr/20 leaves	
		Exp #1	Exp #2
0	Control	2.9	2.8
12	Control	4.2	4.3
12	Irradiated	4.1	4.6
36	Control	3.4	5.1
36	Irradiated	3.4	3.9

Total activities of the successive extracts from the petiole bases are shown in Table 57. No activity was found in the DNA extracts or in the tissue residue. More than 95% of all the activity present in the petiole bases was found in the supernatant after homogenization. Nevertheless, this activity was only a small fraction of that calculated from the volume of uracil-2-C¹⁴ absorbed by the leaves. Presumably, most of the solution absorbed passed up the petiole and into the lamina. No correlation was found between the amount of activity in the supernatant from the homogenized material and that in the RNA extracts. Thus, incorporation of the isotope into RNA did not depend on the total amount of isotope absorbed by the tissue.

TABLE 57

Activity (in counts per minute per extract) recovered from successive extraction steps in the isolation of RNA from petiole bases immersed in uracil-2-C¹⁴

Hours after irradiation	Treatment	Homogenized, supernatant	Ethanol extract	Ethanol:ether extract	Ethanol extract	1st 0.2 N HClO ₄ extract	2nd 0.2 N HClO ₄ extract
0	Control	631,000	36,400	4,120	218	16,600	822
12	Control	677,000	37,100	3,820	413	17,000	793
12	Irradiated	621,000	34,900	3,560	236	16,500	668
36	Control	717,000	46,700	5,020	301	18,600	955
36	Irradiated	751,000	36,900	4,360	380	27,600	1,440

The activity of the nucleotide extracts was over 10 times that of the RNA extracts (compare Tables 55 and 57). There was little difference in activity between the various nucleotide extracts except for those from the 36 hr irradiated leaves; those had much higher activity. Absorbancy spectra (from 220-310 m μ) indicated that the pool sizes were roughly the same. Furthermore, there was no correlation between the activity in the "nucleotide pool" and the incorporation of uracil-2-C¹⁴ into RNA. It may be inferred, therefore, that most of the "pool" was not directly available for RNA synthesis. Since the procedure used did not involve equilibration of C¹⁴-labeled uracil with unlabeled uracil, much of the activity in the "nucleotide pool" might be located in the "apparent free space." (4)

The fact that the incorporation of isotope into the RNA extracts was independent of the amount of isotope absorbed by the tissue or in the "nucleotide pool" substantiates our assumption that the extent of incorporation depended primarily on the rate of RNA synthesis.

We will examine next the actual RNAase activity in the bases of the irradiated lamina. The experiments described in the present report suggest the possibility of a radiation-induced hormonal activation of RNAase activity

References

1. Cherry, J. H., and R. H. Hageman. Nucleotide and ribonucleic acid metabolism of corn seedlings. *Plant Physiol.* 36, 163-168 (1961).
2. Cherry, J. H., R. H. Hageman, and J. B. Hanson. Effect of X-irradiation on nucleic acids in Zea mays. II. On the level of ribonuclease activity in growing seedlings. *Radiation Res.* 17, 740-751 (1962).
3. Passynsky, A. G. Action of radiation on proteins and nucleic acids in solution and at interfaces. *The Initial Effects of Ionizing Radiations on Cells*, ed. R. J. C. Harris. Academic Press, London and New York, 1961. pp 45-57.
4. Briggs, G. E., and R. N. Robertson. Apparent free space. *Annual Review of Plant Physiology*, ed. A. S. Crafts. Annual Reviews, Inc., California, 1957. pp. 11-29.

X-RADIATION RESPONSE OF AUXIN FORMATION BY ANIMAL TISSUE

Solon A. Gordon and Evelyn Buess

Since the classical work of Dale,⁽¹⁾ various studies of protective action indicate that ionizing energies can be dissipated selectively in defined condensed systems. Empirically, however, the dissipation of such energy in non-dormant biological material appears to be defined largely by collision (proximity) probabilities. So few molecules of any one protein species are "hit" during a small number of ionizing events (small with respect to the total number of molecules present) that direct destruction of enzyme molecules in vivo is difficult to detect.

One apparent exception is the radiation response of the auxin-forming enzyme of the higher plant.^(2,3) This enzyme complex, localized in the cytoplasm,⁽⁴⁾ converts tryptophan to the auxin indoleacetic acid (IAA). Exposure of the plant to X-ray doses of <100 rads results in a rapid and sharp depression in activity of the auxin-forming enzyme. This type of response is unusual among enzymes whose activities have been isolated and measured in vitro immediately following irradiation of the organism.

We wondered whether the above radiosensitivity was unique to the plant. Accordingly, we have begun to investigate the tryptophan-auxin enzyme system in animal tissues. This report describes several observations on the enzyme: its distribution, radiation response, and properties associated with that response.

Methods

The term free auxin is here used to mean the net auxin activity in the acid fraction of an ether extract of tissues. For the determination of free auxin, organs that had been frozen in liquid N₂ were coarsely pulverized in the frozen state and placed under diethyl ether at 0-3°C. (All of the ether used was purified by shaking with and distilling from an aqueous suspension of ferrous sulfate-calcium oxide.⁽⁵⁾) The tissues were left under ether for one-half hour at 2°C, the ether then being decanted. This extraction was repeated twice. The pooled ether was reduced in volume to ca. 100 ml, if necessary, and extracted twice with 10-15 ml portions of 2% sodium bicarbonate solution. This aqueous extract was acidified to pH 3 and extracted twice with 2-3 volumes of ether. The ether was then reduced in volume to a few ml by gentle boiling on a steam bath, transferred quantitatively to a 2-inch petri dish, and allowed to evaporate at room temperature. The total auxin activity in the dish was measured biologically by the *Avena* section test,⁽⁶⁾ using the concentration-response curves of crystalline IAA as a reference. It should be emphasized that the value so obtained for free auxin represents not only IAA, but conceivably other auxins, soluble acidic precursors that are converted to IAA by the tissues used for assay, and growth inhibitors.

For measurement of the tryptophan-IAA enzyme concentration, organs were taken from liquid N_2 and finely pulverized while in a frozen state. Weighed samples were homogenized in 2-3 volumes of phosphate buffer, 0.03 M, pH 7.2. The brei was centrifuged at $15,000 \times g$ for 15 minutes. Aliquots of the supernatant solution were used as the enzyme and used also for the determination of trichloroacetic acid-precipitated nitrogen by digestion with $H_2SO_4-H_2O_2$ and direct Nesslerization.

The incubation mixture usually consisted of 0.032 M L-tryptophan, 0.03 M phosphate buffer, pH 7.2-7.3, and 2 ml of the enzyme in a total volume of 10 ml. To stop enzyme activity, 1 ml 10% $ZnSO_4$ followed by 1 ml 0.63 N NaOH and 5 ml of saturated ammonium sulfate solution, neutralized to pH 7, were added. The ammonium sulfate also facilitated phase distribution during partition.

Incubation mixtures were fractionated by extraction with ether at pH 8 and pH 3. Aliquots of the acid fraction were resolved by chromatography on Whatman #1 paper with a solvent system consisting of methanol/n-butanol/0.1 N acetic acid (1:1:98 v/v/v) (cf. Reference 7). The IAA portion of the dried chromatogram, as demarcated on a parallel marker strip, was eluted with methanol and assayed for auxin by the Avena section test. This procedure separated the biological activity of IAA from that elicited by other auxins or inhibitors with migration rates different from that of IAA.

A General Electric "Maximar" was used for X-irradiation. Factors were: 250 kv, 0.5 mm Cu plus 1 mm Al filtration, HVL 1.9 mm Cu, dose rate 200 r per min.

Results and Discussion

Both the enzyme systems that convert tryptophan to IAA, and auxin in the free form, occur in a number of animal organs. Table 58 gives the results of a survey of the dog. The enzyme was found in the testis, liver, kidney, and fibrous muscle, with the testis highest both on a weight and protein basis. No significant enzyme activity was observed for the other organs and glands listed.

Apparently no correlation exists between free auxin and enzyme in the various organs examined. For example, the duodenum and lymph nodes contain appreciable free auxin but little, if any, enzyme activity. This lack of correlation was also observed for the liver, kidney, and testis of various animals (Tables 59 and 60). One of the lowest enzyme titers was that of the newt liver, which possessed the highest free auxin activity encountered.

TABLE 58

Activity of the tryptophan-indoleacetate enzyme and
free acid-auxin levels in various organs of the dog

Tissue	Enzyme Activity						Free Auxin (IAA equivalents), μg/kg tissue
	μg IAA/hr/g tissue			μg IAA/hr/mg N			
	A*	B**	Av.	A	B	Av.	
Testis	7.8	15	11	1.1	1.8	1.4	2
Liver	5.8	5.5	5.6	0.16	0.31	0.24	5
Kidney	4.6	3.8	4.2	0.32	0.20	0.26	1
Muscle (sartorius)	2.1	2.4	2.2	0.30	0.43	0.36	5
Brain				†			†
Heart				†			†
Stomach				†			17
Duodenum				†			30
Lung				†			†
Pancreas				†			5
Spleen				†			2
Thymus				†			5
Hypothalamus				†			20
Pituitary				†			-
Thyroid				†			-
Adrenal				†			†
Blood				†			†
Lymph node				†			50

*Dog A, 35 lb, 3 yr, male.

**Dog B, 26 lb, male.

† Activity below significance

TABLE 59

Enzyme and free acid-auxin activities in the
livers of various young adult animals

	Enzyme Activity		Free Auxin (IAA equivalents), $\mu\text{g/kg}$ tissue
	$\mu\text{g IAA/hr/g}$ tissue	$\mu\text{g IAA/hr/mg N}$	
Hamster	25	1.1	40
Guinea Pig	25	1.0	*
Gerbil	18	0.60	40
Mouse A	19	0.73	-
Mouse B	14	0.51	-
Mouse C	9	0.74	-
Cat	13	0.65	120
Chicken	12	2.2	6
Human	11	0.65	21
Pigeon	11	0.55	20
Rat	8.5	0.42	*
Rabbit	6.5	0.32	16
Sheep	6.0	0.75	*
Dog	5.0	0.24	*
Pig	5.0	0.23	*
Newt	3.5	0.08	360
Beef	3.0	0.12	*

*Activity below significance

TABLE 60

Enzyme and free acid-auxin activities in the kidney
and testis of various young adult animals

	Enzyme Activity		Free Auxin (IAA equivalents), μg , kg tissue
	μg IAA/hr/g tissue	μg IAA/hr/mg N	
<u>Kidney</u>			
Guinea Pig	218	15	*
Hamster	120	3.5	-
Mouse	11	0.63	-
Dog	4.0	0.29	4
<u>Testis</u>			
Hamster	22	2.0	-
Dog	12	1.6	2
Guinea Pig	10	1.0	55
Cat	3.3	0.5	-

*Activity below significance

Of the various livers examined, Table 59 shows that the enzyme concentration is relatively high in the rodent liver, and that the protein of the chicken liver is relatively high in the enzyme. The highest enzyme activity we have encountered was in the kidney of the guinea pig (Table 60) (cf. Reference 8).

Figure 93. Effect of enzyme concentration on the rate of conversion of tryptophan to auxin (mouse liver, tryptophan conc. 0.02 M, pH 7.2, 30°).

The liver of the rodent was used to study the effect of total body X-irradiation on the enzyme system. To evaluate efficiently the effects of irradiation on enzyme titer, we first determined the conditions required for zero-order reaction rates. Figures 93 and 94 demonstrate that the enzyme can be substrate-saturated to yield constant conversion rates. The conversion rate is proportional to enzyme concentration up to ca. 20 mg of precipitable nitrogen per mixture, and the enzyme

Figure 94. Time course of tryptophan conversion to auxin at 30° and 40° (mouse liver, 14 mg N, tryptophan conc. 0.032 M, pH 7.3).

represented by 14 mg of nitrogen yields a constant rate of IAA formation at 30° for at least 3 hours. In the experiments described below, experimental incubations normally involved 2 ml of enzyme preparation and 2 hours at 30° as the incubation time. Incubation times were sometimes three hours for an enzyme of low titer or one hour for preparations with high activity.

Figure 95 describes the effect of total body irradiation on the enzyme in CF No. 1 mouse liver 6 hours after X-irradiation. Activities (micrograms IAA produced per milligram N per hour) of irradiated organs were divided by those from corresponding nonirradiated control animals. The resultant

ratios are plotted for various X-ray doses in the figure. No significant response to 25 r was observed. However, 50 r resulted in a five-fold increase in enzyme activity over respective control livers. The radiation-induced enhancement drops with increasing dose till apparently significant reductions in titer occur in the range of 1 kr and higher.

Figure 95. Effect of various doses of X-radiation on the auxin forming activity of mouse livers. Determinations were made six hours after irradiation and are represented as the ratio of activities of irradiated (m_e) to control (m_c) animals.

We suggest that the radiation-induced rise in enzyme titer is a hormone-mediated stress syndrome. This suggestion is based on the following observations:

First, moving and handling of animals, or the injection of saline solution, generally results in a small increase in the tryptophan-IAA enzyme activity of the liver.

Figure 96. Influence of animal age on the auxin forming capacity of the rat liver

Second, the enzyme activity, on both a weight and nitrogen basis, is a significant function of animal age. Figure 96 indicates that the enzyme titer is high in fetal liver, drops precipitously after birth, and then rises to fetal levels as the animal matures. The span of activities encompasses roughly two orders of magnitude. A similar phenomenon also appears to occur in the rat kidney (Figure 97); here there is also an indication of a minor peak in titer of the kidney, occurring at about 10 days postpartum. The data of Figures 96 and 97 suggest the

possibility that an obligate activator of the enzyme is maternally supplied to the fetus. Self-activation then slowly develops as the animal matures.

Figure 97

Influence of animal age on the auxin forming capacity of the rat kidney

An alternative possibility is the presence of a post-uterine inhibitor of the enzyme, the inhibition slowly decreasing with age.

Third, the enzyme appears amenable to substrate adaptation. Figure 98 shows the effect of intraperitoneal injection of tryptophan on the activity of the enzyme in the mouse liver. The amino acid was introduced at levels of 1 or 3 g/kg body weight. Six or 24 hours after injection the animals were killed by cervical fracture and their livers immediately excised. In the 4 groups of experiments, the activity in the livers of animals receiving tryptophan was higher than that of corresponding saline-injected controls. Enhancement of enzyme activity was observed at both 6 and 24 hours, with a 6- to 7-fold enhancement occurring in one group after 24 hours.

Figure 98. Effect of L-tryptophan, injected intraperitoneally, on the tryptophan-indoleacetate enzyme activity of mouse liver 6 and 24 hours after injection. (0 represents untouched animals, S those receiving saline solution, T those receiving tryptophan in saline at dose levels of 1 and 3 grams per kilogram body weight).

Fourth, adrenalectomy abolishes both the enhancement in titer elicited by low X-ray doses and the reduction in titer that followed exposures in the kr range. Mice were adrenalectomized and given saline solution ad libitum. Two days later they were given a maintenance dose of deoxycorticosterone (1 mg/kg, in sesame oil, subcutaneously). Four days after surgery, groups were irradiated with X-ray doses of 10, 50, 100, 500, and 2,500 rads. Six hours after irradiation the livers were excised for enzyme assay. The data are given in Table 61. None of the irradiated groups yielded activities significantly different from the unirradiated control.

TABLE 61

Tryptophan → IAA activities in livers of adrenalectomized mice 6 hours after X-irradiation

Dose, rads	n	Mean mg N/g tissue	Specific activity, $\mu\text{g IAA/hr/mg N}$
0	12	16.3	$0.13 \pm .02$
10	12	18.1	$0.14 \pm .02$
50	12	17.2	$0.16 \pm .03$
100	12	18.3	$0.11 \pm .01$
500	11	18.3	$0.12 \pm .01$
2500	12	18.6	$0.12 \pm .02$

The experiments described may be summarized as follows:

1. Auxin and the enzyme that converts tryptophan to the auxin IAA are found in many animals though not in all of their organs. Organs relatively rich in the enzyme are the kidney, liver, and testis.
2. The enzyme of the animal is similar to that of the plant in that it responds to exposure of the organism to X-radiation. However, there is a qualitative difference in response. The activity rises in the liver of the mouse after total-body doses on the order of 100 r and is depressed by doses in the kr range. In the plant only inhibition of the enzyme follows single doses of X-radiation.
3. The response of the liver enzyme to X-rays may be interpreted as an indirect endocrine phenomenon: a) there is small stress-induced stimulation, b) the activity in fetal liver (and kidney) is high; it drops at birth and slowly climbs to high titer again in the mature animal, c) the enzyme appears subject to substrate adaptation, d) adrenalectomy abolishes the radiation response.

Some properties of the tryptophan-IAA enzyme are strikingly similar to the tryptophan pyrrolase investigated extensively by Knox⁽⁹⁾ and by Thomson.⁽¹⁰⁾ The pyrrolase is a substrate-adaptable enzyme, under endocrine control of the pituitary and adrenal, whose activity is also enhanced by ionizing radiation. The tryptophan-IAA enzyme system probably does not involve tryptophan pyrrolase for a number of reasons. First, tryptophan pyrrolase activity is not found in higher plants,⁽¹¹⁾ plants that possess the tryptophan-IAA system. Second, kynurenic acid, shikimic acid, prephenic acid, anthranilic acid and indole are not effective as substrates for the formation of IAA by liver preparations. Third, though tryptophan pyrrolase in the rabbit liver progressively increases in activity after birth, the activity in the fetal liver is low.⁽¹²⁾ Fourth, though the pyrrolase enzyme occurs in

liver, its concentration is low in kidney, testis, and muscle, four organs that have relatively high activities of the tryptophan-IAA enzyme. Fifth, the responses of the two enzymes to X-rays appear to be different. The pyrrolase system requires several hundred r for detectable enhancement, doses of 1-2 kr being very effective. The tryptophan-IAA enzyme is enhanced by ca. 50 r, doses in the kr range being inhibitory.

There is also some evidence that the tryptophan-auxin system here dealt with does not directly involve the 5-hydroxy (serotonin) route. First, we are working with IAA rather than 5-hydroxy-IAA; the latter compound, though it has some auxin activity,⁽¹³⁾ is satisfactorily resolved from IAA by the chromatographic technique employed in the present work. Second, inhibitors of amine oxidase activity do not affect the rates of tryptophan conversion to IAA in plant tissues although these inhibitors effectively curtail the oxidation of tryptamine or the formation of auxin from tryptamine.⁽¹⁴⁾ Third, the conversion of tryptophan to 5-hydroxytryptophan has not been encountered in higher animals.⁽¹⁵⁾

References

1. Dale, W. M. The effect of X-rays on the conjugated protein D-amino acid oxidase. *Biochem. J.* 36, 80-85 (1942).
2. Gordon, S. A. and R. Weber. The radiosensitivity of indoleacetic acid. *Plant Physiol.* 30, 200-210 (1955).
3. Gordon, S. A. I. The effects of ionizing radiation on plants: Biochemical and physiological aspects. *Quart. Rev. Biol.* 32, 3-14 (1957).
4. Gordon, S. A. Intracellular localization of the tryptophan-indoleacetate enzyme system. *Plant Physiol.* 33, 23-27 (1958).
5. Larsen, P. Growth substances in higher plants. *Modern Methods of Plant Analysis*. Vol. III, K. Paech and M. V. Tracy, eds., Springer Verlag, Berlin, 1955. pp. 565-625.
6. Bentley, J. A. Analysis of plant hormones. *Methods of Biochemical Analysis*. Vol. 9, D. Glick, ed., Interscience, N. Y., 1962. pp. 75-125.
7. Gordon, S. A. and L. Paleg. Observations on the quantitative determination of indoleacetic acid. *Physiol. Plantarum* 10, 39-47 (1957).
8. Weissbach, H., W. King, A. Sjoerdsma, and S. Udenfriend. Formation of indole-3-acetic acid and tryptamine in animals. A method for estimation of indole-3-acetic acid in tissues. *J. Biol. Chem.* 234, 81-86 (1959).

9. Knox, E. Two mechanisms which increase in vivo the liver tryptophan peroxidase activity: Specific enzyme adaption and stimulation of the pituitary adrenal system. *Brit. J. Exptl. Pathol.* 32, 462-469 (1951).
10. Thomson, J. F. and E. T. Mikuta. Effect of total body X-irradiation on the tryptophan peroxidase activity of rat liver. *Proc. Soc. Exptl. Biol. Med.* 85, 29-32 (1954).
11. Wiltshire, G. H. Oxidation of tryptophan by pea-seedling tissues and extracts. *Biochem. J.* 55, 408-416 (1953).
12. Nemeth, A. M. Initiation of enzyme-formation by birth. *Science* 132, 1497 (1960).
13. Ek, A. and B. Witkop. Synthesis and biochemistry of 5- and 7-hydroxy-tryptophan and derivatives. *J. Am. Chem. Soc.* 75, 500-501 (1953).
14. Gordon, S. A. Biogenesis of natural auxins. *Chemistry and Mode of Action of Plant Growth Substances*, ed. R. L. Wain and F. Wightman. Butterworths, London, 1956.
15. Ichihara, K., A. Sakamoto, K. Insmori, and Y. Sakamoto. Studies on the acid diazo reaction on 5- or 7-hydroxyindole derivatives. *J. Biochem. (Tokyo)* 44, 649-659 (1957).

SPECTROPHOTOMETRIC METHOD FOR THE DETERMINATION OF LIPOXIDASE ACTIVITY

Kenneth Surrey

Holman and Burr⁽¹⁾ and Bergström⁽²⁾ independently observed an increase in ultraviolet light absorption when lipoxidase acted upon essential fatty acids. The increase in peak absorption (234 m μ) was then related to peroxide formation, which was found to be proportional to time and enzyme concentration.^(3,4) Although satisfactory results are obtained when the test is applied at pH 9.0 or above, where the unsaturated fatty acids are present in a soluble form, this nonphysiological pH is the main disadvantage of the spectrophotometric method. It was, therefore, concluded by Holman and Bergström⁽⁵⁾ that the effect of pH on the inherent activity of the enzyme could not be determined until a water-soluble substrate could be found.

The method described here is essentially a modification of the methods of Theorell et al.⁽⁴⁾ and Tappel.⁽³⁾ The polyunsaturated fatty acid (linoleate) is solubilized by the addition of a detergent, and the activities of purified and crude lipoxidase are demonstrated over a wide range of pH, using the homogeneous substrate.

Materials and Methods

The experimental material consisted of soybean meal (Glycine max L.) or gram flour (Cicer sp.) which had been washed previously with petroleum ether and stored in a refrigerator. Lipoxidase activities were also measured in hydrated mung beans (Phaseolus aureus L.), and Brassica and lettuce seeds. The material was ground with sand and water or phosphate buffer. Each homogenate was brought to the required pH before centrifugation at 2000 x g for 15 minutes. The supernatant liquid was used as the enzyme source. Linoleic acid and purified lipoxidase were obtained from Nutritional Biochemicals Corporation.

For routine assay, substrate was prepared as follows: One-half ml of Tween 20 was dissolved in 10 ml of borate buffer at pH 9.0, and 0.5 ml of linoleic acid was added drop by drop. The contents were thoroughly mixed to form a fine emulsion. Then 1.3 ml of 1 N NaOH were added and the mixture was once again agitated until a clear, transparent solution was obtained. To this solution 90 ml of borate buffer were added and the final volume made to 200 ml with water. The resulting substrate solution (0.25%), approximately 7.5×10^{-3} M in linoleic acid, was adjusted to the desired pH with concentrated HCl.

Mixtures containing measured volumes of enzyme and substrate were incubated at room temperature and were continuously oxygenated. A

1-ml sample of the incubation mixture was transferred periodically into each of several 12-ml centrifuge tubes containing 2 ml of absolute ethanol. To each tube 7 ml of 60% ethanol were later added to make a total volume of 10 ml. Tubes were centrifuged if necessary. Optical densities of clear alcoholic solutions were read at $234\text{ m}\mu$, against a blank, on a Cary recording spectrophotometer, Model 11.

The blank solution was prepared using volumes of substrate and enzyme equal to those used in the test samples. Enzyme was added to 2 ml of absolute ethanol. After mixing, the tube was allowed to stand for a few minutes. Sixty per cent ethanol was then added, followed by substrate. If necessary, further dilutions of test and blank samples were made with 60% ethanol.

A unit of lipoxidase is defined as that activity which will produce an optical density of 1, at $234\text{ m}\mu$, in a total volume of 10 ml of 60% ethanol solution.

Results

Purified lipoxidase

The effect of pH on the activity of purified enzyme is shown in Figure 99. Top curve shows the optimum pH to be 7.0, with the activity falling

Figure 99

Effect of pH on the activity of purified lipoxidase. Top curve, with Tween 20; lower curve, without Tween 20. One mg of enzyme was dissolved in one ml of each of the graded series of phosphate buffers, and this solution was mixed with 19 ml of linoleate solution (with or without Tween 20) at the same pH.

off symmetrically on either side of this point. However, when Tween 20 was omitted from the substrate solution (lower curve), three changes were noted: The pH optimum was shifted to 7.5; activities were much lower over the entire pH range, except at pH 9.0 where the difference between the two points tends to disappear; and the activities declined abruptly below pH 7.5.

Figure 100. Effect of pH on the activity of crude lipoxidase of soybean meal. Enzyme was extracted from 1 g of meal with 200 ml of phosphate buffer at the desired pH; 1 ml of this extract was incubated with 9 ml of linoleate plus Tween 20 solution at the same pH. Activities were determined over a period of 6 min.

Tween 20). In both substrate solutions the pH optimum of the enzyme was 6.5. Virtually no activity was observed on either side of the physiologically active pH range (5 to 7.5).

Mung beans

pH effect. In Figure 102 lipoxidase activity is plotted as a function of pH, with varying periods of incubation. It can be seen that the enzyme had maximum activity at pH 5.5 and that activity dropped sharply on the acid side. On the alkaline side, activity declined more gradually, but no activity was observed above pH 7.5.

When the amount of detergent was increased from 0.25 to 0.5%, linoleic acid concentration being constant (0.25%), the enzyme activity was inhibited and the pH optimum shifted to 6.5. Since no ultraviolet light absorption was recorded when Tween 20 alone was used as substrate, the detergent itself does not appear to serve as substrate for the system under investigation.

Soybean meal

The effect of pH on crude lipoxidase of soybean meal is shown in Figure 100. Optimum pH is about 6.0. The activity declined sharply on the acid side; on the alkaline side, the activity declined gradually up to pH 7, fell sharply between 7.0 and 7.5, and leveled off beyond pH 8.0.

Gram flour

The effect of pH on gram flour lipoxidase is shown in Figure 101 (top curve with, and lower curve without,

Figure 101

Effect of pH on the oxidation of linoleate by crude lipoxidase from gram flour. Top curve, linoleate and Tween 20. Lower, linoleate only. Lipoxidase from 1 g of gram flour was extracted in 50 ml of each of the graded series of phosphate buffers; 1 ml of the extract was mixed with 9 ml of the substrate at the same pH.

Figure 102

Effect of pH on the oxidation of linoleate by crude lipoxidase of hydrated mung beans. Sixteen g of mung bean seeds hydrated for about 18 hr were ground in sand and in 200 ml of water. The water extract was centrifuged and divided into several portions, each of which was adjusted to a desired pH with minimal quantities of HCl or NaOH. The extracts were recentrifuged, and the supernatant liquid used as the enzyme preparation. One ml of enzyme solution was mixed with 19 ml of substrate solution at the same pH.

Effect of substrate concentration. As mentioned above, increase in Tween 20 concentration, in a fixed amount of linoleate, causes partial inhibition of the oxidative capacity of purified lipoxidase. In the present experiment the effect of increasing the concentrations of both Tween 20 and

linoleate by the same amount was determined. The results, measured at various incubation times, are shown in Figure 103. The highest activity was found at 0.5% substrate concentration for the first 6 min of incubation. At 7 min incubation the activities of 0.5% substrate and 0.25% substrate were about the same and between 7 and 10 min the highest activity was found at the lowest substrate concentration, 0.25%. At the 1% substrate level, the activity was initially lower than at the 0.5% level, and for the first 4 min of incubation matched closely that at 0.25%. A slight increase in activity, relative to the 1% concentration, was seen at the 1.5 and 2.0% levels during the first 2 min of oxidation, but the activities leveled off much earlier than those of the lower substrate concentrations. It is thus apparent that the optimal substrate concentration is about 0.25 to 0.5% and that increase in substrate concentration results in decreased enzyme activity. It should be pointed out that this experiment does not discriminate between differential inhibitions caused by the individual components of the substrate, but rather illustrates the cumulative effect of varied substrate concentrations.

Figure 103

Effect of substrate concentration on linoleate oxidation by crude lipoxidase of hydrated mung beans. The concentrations of linoleate and Tween 20 were increased by the same amount and the pH of each substrate solution was adjusted to 5.5. Enzyme was extracted in 25 ml water per gram of hydrated seed and the pH adjusted to 5.5 before centrifugation. One ml of enzyme solution was mixed with 19 ml of substrate solution.

Other seeds

Similar tests were made on dry and hydrated seeds of turnip, radish, mustard and lettuce seeds. These preparations did not oxidize linoleic acid.

Discussion and Summary

The results of this investigation show that the optimum pH of lipoxidase depends upon the source from which the enzyme is obtained. Although most of the experimental materials reported here were of leguminous origin, they differed in their response to pH. For purified lipoxidase and crude enzymes prepared from gram flour, soybean meal, and hydrated mung beans, pH optima were 7.0, 6.5, 6.0 and 5.5, respectively.

The presence of Tween 20 facilitates uniform distribution of fatty substrates, so that pH can be varied extensively without the formation of an emulsion. Tween 20 at the standard concentration did not interfere with the lipoxidase catalysis and did not absorb ultraviolet light. Its incorporation in fatty substrate consistently contributed to higher lipoxidase activity (Figures 99 and 101, upper curves). Further evidence of its noninterference comes from Holman (cited in Reference 5) who observed that Tween prepared from linoleic acid and polyoxyethylene-sorbitan was not attacked by lipoxidase. It should be pointed out that in the absence of Tween 20 (lower curves of Figures 99 and 101), there was no accelerated enzymatic activity at pH 9.0 and above, as reported by the original authors.⁽³⁻⁵⁾ Thus, the present modified spectrophotometric method can be applied without the inherent limitations described earlier.

Since linoleic acid is a constituent of naturally-occurring fatty substances of Brassica seeds⁽⁶⁾ and of lettuce seeds (Meara, cited in Reference 7), their failure to oxidize linoleate may be related to their antioxidant content. The presence of antioxidants, such as tocopherols, has been reported in cruciferous⁽⁸⁾ and other seeds.⁽⁹⁾ Similarly, germination inhibitors are present in Brassica and lettuce seeds.⁽¹⁰⁾ Whether these two categories of naturally occurring substances have similar physiological functions is not known.

References

1. Holman, R. T. and G. O. Burr. Spectrophotometric studies of the oxidation of fats. IV. Ultraviolet absorption spectra of lipoxidase-oxidized fats. *Arch. Biochem.* 7, 47-54 (1945).
2. Bergström, S. On the oxidation of linoleic acid with lipoxidase. *Arkiv Kemi Mineral Geol.* 21A (15), 1-8 (1946).
3. Tappel, A. L. Lipoxidase. *Methods in Enzymology*, Vol. V, ed. S. P. Colowick and N. O. Kaplan. Academic Press, New York, 1962.
4. Theorell, H., S. Bergström, and Å. Åkeson. Activity determination and further purification of lipoxidase. *Pharm. Acta Helv.* 21, 318-324 (1946).

5. Holman, R. T. and S. Bergström. Lipoxidase or unsaturated-fat oxidase. The Enzyme Chemistry and Mechanism of Action, Vol. II, Part I, ed. J. B. Sumner and K. Myrbäck. Academic Press, New York, 1951.
6. Hilditch, T. D., T. Riley, and N. L. Vidyarthi. The fatty acids of seed oils of Brassica species. The composition of rape, revision, and mustard seed oils. J. Soc. Chem. Ind. 46, 457-468 (1927).
7. Brown, H. J. M and J. Thick. Effects of seed extracts on radiosensitivity. Proc. Symp. Effects of Ionizing Radiation on Seeds, Karlsruhe, Germany, Internatl. Atomic Energy Agency, Vienna, Austria, 1961. pp. 75-80.
8. Janicki, J., A. Rutkowski, J. Furyk, T. Kulik, and W. Olszewski. Antioxidative capacities of seeds of some native and introduced plants. Roczniki Tech. Chem Zywnosci. 1, 85-97 (1957).
9. Green, J. The distribution of tocopherols during the life-cycle of some plants. J. Sci. Food Agric. 9, 801-812 (1958).
10. Toole, E. H., S. B. Hendricks, H. A. Borthwick, and V. K. Toole. Physiology of seed germination. Ann. Rev. Plant Physiol. 7, 299-324 (1956).

PROGRESS REPORT: THE EFFECT OF X-RAYS ON THE FORMATION OF FLOWER PRIMORDIA IN XANTHIUM

William Chorney

Most of our theories on flowering in plants have been based on experimental evidence gathered using *Xanthium* as the test organism. It is the most sensitive of the short-day plants. It will readily form flower primordia if a single leaf is subjected to one dark period whose duration exceeds the critical photoperiod.⁽¹⁾ The most sensitive single leaf in initiating the flowering response in *Xanthium* is the uppermost, when 1/2 to 3/4 expanded.⁽²⁾

A number of processes must follow in succession in order for flower primordia to be formed by one inductive cycle. Two high-intensity light requirements have been recognized. The first, which precedes the inductive dark period, is responsible for the synthesis of precursors of the flowering stimulus formed by the inductive dark period. The stimulus is stabilized in the second light period and is translocated to a stem meristem where it triggers the initiation of flower primordia.⁽³⁾ This program was undertaken to determine whether low levels of X-rays applied to the inducing leaf during one of light periods would alter the flowering response in *Xanthium*.

Xanthium plants were grown on an 18-hour photoperiod until the leaf at the 6th node was about 3/4 expanded. Plants of uniform size were selected and trimmed to a single leaf at the 6th node. In all experiments only the leaf used for photoperiodic induction was X-irradiated, the terminal meristem being shielded by at least 1/4 in. of lead. A GE Maximar X-ray machine, operated at 250 kv, 15 ma, with no added filtration, was used. Dose rate was 50 r per minute. After the inductive cycle the plants were returned to the 18-hour photoperiod for 10 days, at which time the terminal meristem was dissected to determine the stages of floral development.⁽²⁾

Irradiation of the leaf with 2.0 kr in the first light period after the inductive night had a slight inhibitory effect on flowering; 3.0 kr prevented the formation of flower primordia. When the X-rays were given in the second light period, i.e., 24 hours, after the end of the inductive night, no effect on flowering was found.

X-irradiation of the leaf by doses of 0.25 kr to 5 kr prior to the inductive night stimulated the production of flower primordia. Saturation was reached at 1 kr, and the same response was maintained up to 5.0 kr. Gibberellic acid (1.0 mg/1.), applied to the inducing leaf by a 20-second dip, prior to the inductive night but either before or after X-irradiation,

enhanced the stimulatory effect of the X-rays. Gibberellic acid alone had no effect on flowering. X-irradiation produced no macroscopic morphological changes in the leaf and did not affect the growth rate of the terminal meristem.

These preliminary results indicate that low levels of X-rays applied to the inducing leaf can alter the flowering response in *Xanthium*. Flowering is stimulated when the leaf is exposed to X-ray prior to the inductive dark period, inhibited when the leaf is exposed during the first post-inductive light period.

Several interpretations of these effects of X-rays may be suggested. Inhibition might result from interference with the translocation of the flowering stimulus, degradation of the stimulus, or production of substances that inhibit the action of the stimulus at the meristem. The stimulatory effect could result from shortening of the critical night (allowing for the production of more flowering stimulus by a given exposure), or from lowering the concentration of an inhibitor. These possibilities are being investigated.

References

1. Hamner, K. C. and J. Bonner. Photoperiodism in relation to hormones as factors in floral initiation and development. *Botan. Gaz.* 100, 388-431 (1938).
2. Salisbury, F. B. The dual role of auxin in flowering. *Plant Physiol.* 30, 327-334 (1955).
3. Doorenbos, J. and S. J. Wellensiek. Photoperiodic control of floral induction. *Ann. Rev. Plant Physiol.* 10, 147-184 (1959).

PROGRESS REPORT: THE BORON REQUIREMENT
OF CHLORELLA VULGARIS

Landy J. McBride

It has long been known that higher plants require boron as an essential mineral nutrient, though we have little knowledge of its physiological role. Unicellular green algae would be particularly useful in the study of the physiology of boron. However, it has never been shown that green algae require this microelement. The literature on the subject is contradictory, and in no instance has the experimental approach been augmented by analysis of the levels of boron present in the systems studied.

This project, begun in September, 1961, was designed to continue and extend the work of McIlrath and Skok,⁽¹⁾ which provided evidence that the growth of Chlorella vulgaris was enhanced by the addition of 0.5 ppm boron to the culture medium. In particular, it was planned to study the effect of boron on carbohydrate nutrition and photosynthesis.

In McIlrath and Skok's original work boron-free glass Erlenmeyer flasks were used for culture vessels. The cultures were agitated on a shaking machine under moderate light intensity. This system needed improvement for two reasons. First, only initial and final cell counts could be obtained, so that precise definition of the growth rate of the organism was difficult. Secondly, only limited amounts of cell material were available for chemical analysis and experiments.

The first project completed was the assembly of an apparatus that provided better growth conditions, permitted growth rates to be estimated, and yielded reasonable amounts of cell material. The apparatus consisted of individual culture vessels made from boron-free glass bottles of one-liter capacity. Each culture was stirred by a teflon-covered magnet, motor-driven from below. Both sides were illuminated by 40-watt (4500° K) fluorescent tubes. The cultures were aerated continuously with hydrated air containing five per cent carbon dioxide. This aeration system contributed no measurable boron to the system.

For the first experiment, Chlorella cells which had been subcultured for several months on minus boron medium were isolated and washed free of bacteria. These cells were subcultured three times on minus boron medium in boron-free Erlenmeyer flasks, and were used as inocula for cultures in the liter bottles. After significant growth had taken place, a second identical set of cultures was established using cells from minus boron cultures of the first set, and similarly a third set of cultures was established from the second. Added boron did not increase the growth rate nor did it increase dry weight per cell.

Since it was possible that the apparatus used in the work of McIlrath and Skok was in some way uniquely suitable for demonstrating a boron effect, an experiment was done with the shaking machine and Erlenmeyer flasks using boron-depleted cells as the inoculum. No significant boron effect was observed in successive duplicate experiments.

In an additional experiment, magnesium sulfate which had been purified by evaporation of contaminating boron as the methyl ester was used in the nutrient medium. The *Chlorella* cells did not respond to added boron.

At this point it was postulated that continued subculturing on minus boron medium had perhaps resulted in a population of *Chlorella* cells which had little or no requirement for boron. A slant of the original "wild type" *Chlorella* was obtained. The cells did not show a response to added boron either in shake culture or in aerated one-liter cultures.

From this point on the work became a problem in analytical chemistry. If a boron effect is lacking because of contaminated distilled water and nutrient salts, these materials must be purified. Further, the boron content of the cells must be determined. Even if a boron effect is eventually not shown, a "balance sheet" on all boron in culture medium and cells is necessary.

Initial boron analyses were carried out using a modified curcumin-oxalic acid method.⁽²⁾ A solution of the reactants is evaporated on a constant-temperature water bath, and the red reaction product between boron and curcumin is taken up in alcohol. The boron content is determined spectrophotometrically at 550 m μ . High salt contents interfere in this method, and traces of nitrate ion abolish color formation.

Nutrient salts (single or mixed) could not, therefore, be analyzed directly. An all-quartz unit for distillation of boron as the methyl ester was introduced. With this apparatus, analyses of all nutrient salts (except the nitrates) were possible. However, the amounts of boron found were rather low, and the procedure was lengthy and unreliable. Nitrate was carried over in the distillate so that nitrate salts could not be analyzed by this method. Several modifications of the technique did not resolve the problem.

Boron in distilled water could be concentrated by evaporation with alkalis such as calcium hydroxide. However, the method was not sensitive enough to obtain reliable analyses of distilled water. Again, several modifications were tried without success. Several ion exchange resins were tested but apparently did not remove boron.

At this point it was found that the porcelain dishes used to evaporate water samples gave off boron into the hot, alkaline solutions. Platinum dishes gave more reproducible results. Furthermore, we found that the curcumin method was unreliable; several modifications proved ineffective.

Two new developments have done much to resolve the above problems. First, evaporating dishes of polypropylene plastic have become available and, except for ashing, serve as well as platinum. Second, a new method for determining boron, based on curcumin dye, but avoiding evaporation during color development, has recently appeared.⁽²⁾ With these techniques we found that the distilled water employed for media contained about 2 micrograms boron per liter.

Several attempts were made to obtain boron-free water by redistillation from an all-quartz unit. Reduction in boron concentration was achieved, but the method was slow and unreliable.

The use of ion exchange resins to remove boron from distilled water was re-examined. We found that "boron-free" water can be obtained by passing distilled water first through a long column of Amberlite MB-3 (anion and cation exchange resin mixed), and then through a column of Permutit S-2 in the hydroxyl form.

With the above resins, we have developed a technique to separate boron from other interfering ions. It permits rapid analyses of nutrient salts and salt mixtures. A salt solution is first passed through a small column of Dowex 50 in the sodium form. The effluent is then passed through Dowex 1 in the formate form. The final effluent contains sodium formate and borate. The solution is made alkaline and evaporated to dryness and the boron analysis is carried out on the dry cake. This method is being tested to determine its reliability and reproducibility. *Chlorella* cultures will again be grown to determine if a boron essentiality can be shown.

The helpful advice of Mr. W. Chorney is gratefully acknowledged.

References

1. McIlrath, W. J. and J. Skok. Boron requirement of *Chlorella vulgaris*. *Botan. Gaz.* 119, 231-233 (1958).
2. Hayes, M. R. and J. Metcalfe. The boron-curcumin complex in the determination of trace amounts of boron. *Analyst* 87, 956-969 (1962).

PUBLICATIONS

July 1, 1962 through December 31, 1962

- Anderson, R. W. and P. E. Gustafson. Concentration of cesium-137 in human rib bone. *Science* 137, 668 (August 31, 1962).
- Bray, D. A. and J. F. Thomson. Effect of deuterium oxide incorporation of P^{32} into rat liver deoxyribonucleic acid. *Proc. Soc. Exptl. Biol. Med.* 111, 380-383 (November 1962).
- Frigerio, N. A. and B. R. Nebel. The quantitative determination of osmium tetroxide in fixatives. *Stain Technol.* 37(6), 347-350 (November 1962).
- Frigerio, N. A. and P. J. Trotter. Use of syringes in gas-law experiments. *J. Chem. Ed.* 39, 594 (November 1962). Letter.
- Frigerio, N. A. Removal of $SbOCl$ films from cuvettes used in the Carr-Price determination. *Chemist-Analyst* 51, 82-83 (October 1962).
- Frissel, M. J. and W. E. Kisielecki. The simultaneous determination of C^{14} and H^3 in biological material, using the Schoniger combustion technique. Institute for Atomic Sciences in Agriculture, Wageningen, Netherlands, Report No. 3, 1962.
- Gustafson, P. F., S. S. Brar and M. A. Kerrigan. Airborne radioactivity due to nuclear weapons tests. *J. Geophys. Research* 67, 4641-4651 (1962).
- Gustafson, P. F., R. B. Keener, G. A. Mc Ginnis and R. A. Soller. Fission product radioactivity in the north polar stratosphere. *Science* 138, 433-434 (October 19, 1962).
- Gustafson, P. F. Estimates of current and future radiation dose to man from nuclear weapons testing through 1961. *Health Phys.* 8, 463 (August 1962). Abstract.
- Gustafson, P. F., S. S. Brar and U. C. Mishra. Determination of airborne fission product radioactivity using gamma-ray spectrometry. *Nature* 195, 557-559 (1962).
- Jaroslow, B. N. and W. H. Taliaferro. Restoration of hemolysin-forming capacity in irradiated rabbits. *Proc. Symp. Effects of Ionizing Radiation on Immune Processes*, Univ. Kansas, Lawrence, September 6-8, 1961. New York: Gordon and Breach Science Publs., Inc., 1962. pp. 301-313.

PUBLICATIONS (Contd.)

- Kisieleski, W. E. and Renato Baserga. Recent observations on cell proliferation and metabolism by radioautography with tritiated thymidine. *Atompraxis* 8(10), 386-391 (October 1962).
- Klein, P. D. and P. A. Szczepanik. The differential migration of sterol acetates on silica gels and its application to the fractionation of sterol mixtures. *J. Lipid Research* 3, 460-466 (1962).
- Kubitschek, H. E. Discrete distributions of generation rate. *Nature* 195, 350-351 (July 28, 1962).
- Lisco, Hermann, Renato Baserga and W. E. Kisieleski. Further observations on induction of tumors in mice with radioactive thymidine. *Proc. Soc. Exptl. Biol. Med.* 110(4), 687-690 (August-September 1962).
- Nebel, B. R. and E. M. Coulon. Enzyme effects on pachytene chromosomes of the male pigeon evaluated with the electron microscope. *Chromosoma* 13, 292-299 (1962).
- Nebel, B. R. and E. M. Coulon. The fine structure of chromosomes in pigeon spermatocytes. *Chromosoma* 13, 272-291 (1962).
- Pfeffer, Morris and S. K. Shapiro. Biosynthesis of methionine from S-adenosylmethionine in *Escherichia coli*. *Biochem. Biophys. Research Commun.* 9(5), 405-409 (November 1962).
- Powers, E. L. Considerations of survival curves and target theory. *Physics in Medicine and Biology* 7(1), 3-28 (July 1962).
- Sacher, G. A. and E. Trucco. A theory of the improved performance and survival produced by small doses of radiations and other poisons. *Biological Aspects of Aging. Proc. 5th Intern. Congr. Gerontology*, ed. M. W. Shock. Columbia Univ. Press, 1962. pp. 244-251.
- Salvatore, F. and Fritz Schlenk. A new assay of guanidinoacetate methyltransferase. *Biochim. Biophys. Acta* 59, 700-702 (1962).
- Schlenk, Fritz and J. L. Dainko. The alkaline hydrolysis of S-adenosylmethionine in tritiated water. *Biochem. Biophys. Research Commun.* 8(1 and 2), 24-27 (June 1962).
- Schubert, Jack and W. M. Westfall. Effects of ionizing radiation and peroxides on hemocyanin. *Nature* 195, 1096-1097 (September 15, 1962). Letter.

PUBLICATIONS (Contd.)

- Skok, John. Further aspects of the photoperiodic responses of Sequoia gigantea. Bot. Gaz. 124, 17-19 (September 1962).
- Slatis, Herman and B. E. Ginsburg. The use of purebred dogs in research problems of genetics. Proc. Animal Care Panel 12(4), 151-155 (September 1962).
- Stearner, S. P. and S. A. Tyler. Comparative aspects of acute radiation mortality in birds. Intern. J. Radiation Biol. 5, 205-215 (July 1962).
- Stroud, A. N., B. R. Svoboda, A. M. Brues and G. O. McDonald. Utilization of thymidine as an indicator of drug effect upon cancer cells. Proc. Surgical Forum 13, 99-101 (1962).
- Surrey, Kenneth. Action and interaction of monochromatic light on phosphate metabolism of germinating lettuce seeds. Can. J. Botany 40(7), 965 (July 1962).
- Svihla, George and Fritz Schlenk. Ultraviolet photomicrography of yeast cells. Proc. Intern. Microscopy Symp., Chicago, June 13-15, 1960, ed. W. C. McCrone. Chicago: McCrone Associates, 1962. pp. 104-106.
- Svoboda, B. R., A. N. Stroud, H. B. Haley, Jr. and W. Wellband. Relationships between chromosomes and biological behavior of two strains of transplantable human tumor H.Ad.1. Proc. Surgical Forum 13, 115-117 (1962).
- Taliaferro, W. H. and L. G. Taliaferro. The relation of antigen dosage to the Forssman hemolysin response in rabbits. Science 138, 994 (November 30, 1962). Abstract.
- Taliaferro, W. H. Remarks on the immunology of leishmaniasis. Scientific Reports of the Instituto Superiore di Sanita 2, 138-142 (1962).
- Thomson, J. F. and J. J. Darling. A note on the mechanism of action of rabbit muscle lactate dehydrogenase. Biochem. Biophys. Res. Commun. 9, 334-336 (1962).
- Thomson, J. F., D. A. Bray and J. J. Bummert. Effects of deuterium on malic dehydrogenase. Biochem. Pharmacol. 11, 943-948 (1962).

PUBLICATIONS (Contd.)

- Thomson, J. F. and Y. E. Rahman. Effect of X-radiation on distribution and properties of cytoplasmic particulates of rat liver. *Radiation Res.* 17, 573-578 (October 1962).
- Thomson, J. F., R. L. Straube and D. E. Smith. The effect of hibernation on liver regeneration in the ground squirrel (Citellus tridecemlineatus). *Comp. Biochem. Physiol.* 5, 297-305 (1962).
- Tyler, S. A. and M. H. Dipert. On estimating the constants of the 'multi-hit' curve using a medium speed digital computer. *Phys. in Med. and Biol.* 7(2), 201-212 (October 1962).
- Vogel, H. H., Jr. and D. L. Jordan. Fertility of female mice irradiated with fission neutrons. *Radiology* 79, 120-125 (July 1962).
- Yall, Irving. Biosynthesis of S-adenosylmethionine by Saccharomyces cerevisiae. I. The adenine and methionine requirements. *J. Bacteriol.* 83, 1336-1340 (June 1962).
- Yanders, A. F. and R. K. Seaton. The lack of mutagenicity of caffeine in *Drosophila*. *American Naturalist* 96, 277-280 (1962).