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BIOLOGICAL AND MEDICAL RESEARCH DIVISION

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1964

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THE INTERRELATIONSHIP BETWEEN CYTOLOGY AND PHYSIOLOGICAL FUNCTION OF THE YEAST CELL

Richard E. Alper

PURPOSE AND METHODS

During the last decade, chemistry and microbial physiology have moved into a stage of development that aims at clarification of the interrelationships between structure and function. The investigator is no longer satisfied with mere description of biochemical reactions, their quantitative assay, and investigation of the enzymes. It is now mandatory to include in such studies the features of intracellular structure, metabolic compartmentalization, membrane effects, and the translocation of metabolites.

While considerable success has been attained in this respect with certain types of mammalian cells, especially liver, there is much less research on yeast cells. This material offers many unique features and advantages for biochemical studies, and its neglect compared with tissue cells may be due to technical difficulties encountered in yeast cytology. Experimentation has been initiated, therefore, to overcome these difficulties.

The conventional repertoire of biochemistry was augmented in this study by ultraviolet and electron microscopy. The former permits localization of ultraviolet-absorbing cellular constituents *in vivo* without fixing, staining, or other preparatory steps that might cause artifacts. However, the resolving power in ultraviolet microscopy, though somewhat better than that of conventional light microscopy, is not sufficient to yield information on many details of fine structure. It was endeavored, therefore, to carry out electron microscopy on the same cell material and to correlate the observations obtained by these two methods. Related chemical analyses were performed to provide quantitative data on cellular components under various culture conditions.

The yeasts *Saccharomyces cerevisiae* and *Candida utilis* were used. These are by far the most frequently used types of yeast, and the results of the present work promised to illuminate many observations of workers in the past. *S. cerevisiae*, moreover, can be induced to

sporulate. Details of the sporulation process are almost entirely unknown.

Two types of enzyme preparation were employed to act on the cell wall and membranes: the complete array of enzymes from the digestive juice of *Helix pomatia* fortunately proved suitable for disintegrating the yeast cell walls, and pancreatic ribonuclease was found very effective in treating cellular membranes.

PROGRESS REPORT

Ribonuclease was found to attack the yeast cell membrane, presumably by fission of the internucleotide bonds adjoining pyrimidine bases. The immediate release of copious amounts of ultraviolet-absorbing intracellular components proved that the action of the enzyme was not restricted to the ribonucleic acid that is a constituent part of the cell membrane.

Ultraviolet microscopy of the cells showed a general increase in transmittance of the cytoplasm at 260 m μ . Electron microscopy of cells after the enzyme treatment gave the following picture. No major lesions in the cell membrane could be discovered. It must be assumed, therefore, that the openings created by enzyme action are very few or very small. The organization of the cytoplasm was greatly disturbed. The nuclear and the vacuolar membranes were severely damaged. The mitochondrial structures, however, remained unaltered in appearance.

Not all of these effects are necessarily caused by enzymatic action. Osmotic shock appears to contribute heavily to the damage. This was treated by carrying out the incubation with ribonuclease in isotonic media such as 1.0M mannitol and sucrose. This minimizes internal destruction, but the mitochondria are less well preserved under these conditions.

Experiments with spheroplasts provided a more detailed study of the cell membrane. Exposure to the ribonuclease led to the internal changes that had been observed in the intact cells. The cytoplasmic membrane,

however, could now be studied in more detail. It did not show major lesions; yet the release of intracellular constituents after action of the enzyme proves that the effect is not merely an alteration of permeability. Viability of the cells is lost after contact with the ribonuclease.

STUDIES ON NUCLEAR PROTEINS

A. D. Barton

PURPOSE AND METHODS

These studies on nuclear proteins stem from a long-term interest in the functions of the cell nucleus. Much of the previous work in this project has dealt with a class of soluble proteins that are retained when rat liver cell nuclei are isolated in sucrose media, and are liberated when the isolated nuclei are extracted with an ionic medium, such as isotonic saline. These "saline-soluble" proteins of the nucleus are relatively basic compared with the soluble proteins of the cytoplasm, though not so basic as histones. They are retained in the nucleus by ion binding, and their behavior in moving boundary electrophoresis changes with changes in the physiological status of the animal.⁽¹⁾ More recently, these studies have been extended to include investigation of other types of nuclear protein under conditions in which the metabolic condition of the animal is altered: during enlargement of liver cell nuclei with thioacetamide administration, for example, and during enlargement of kidney cell nuclei with testosterone administration.

PROGRESS REPORT

The experiments with thioacetamide and testosterone were carried out with the aid of Associated Colleges of the Midwest semester students E. A. Cerny and K. M. Tracy; a preliminary report of the results has been presented^(2, 3) and a paper dealing with the results published.⁽⁴⁾ These experiments suggest the following interesting conclusions, in addition to those presented previously.

The conclusion of Mirsky and Ris that the nuclei of cells with more metabolically active cytoplasm contain more "residual" protein has been confirmed and extended by our finding that rat liver cell nuclei contain more saline-soluble protein, more residual protein, and more RNA, per mg of DNA, than do rat kidney cell nuclei. During differentiation, the capacity of the liver cell nuclei to bind the saline-soluble proteins is adjusted more or less permanently to a level appropriate for liver (and quite different from that in kidney, for

CONCLUSIONS

From the present data it is apparent that a new type of cell ghost has been obtained which offers interesting prospects of differential staining and extraction of remaining constituents, such as enzymes and nuclear materials.

example). However, in changing from quiescence to active gene expression in the liver, there is no change in the quantity, but there is a marked change in the electrophoretic behavior of the saline-soluble proteins.⁽¹⁾ The experiments with testosterone suggest that the concentration of the soluble proteins that are not actually bound in the nucleus is determined by equilibration with corresponding proteins in the cytoplasm.

In work reported earlier,⁽⁵⁾ we found that when active metabolism and synthesis of cell constituents was induced in the livers of animals previously quiescent, characteristic peaks appeared in the electrophoretic patterns of the saline-soluble nuclear proteins.⁽¹⁾ This interpretation was confirmed more directly by our finding that the characteristic peaks (particularly the one with mobility $-0.6 \text{ cm}^2 \times 10^{-5} \text{ v}^{-1} \text{ sec}^{-1}$) appear in the electrophoresis patterns when enzyme synthesis is induced in the liver by administration of hydrocortisone.⁽⁶⁾ Work in other laboratories has shown that the action of a number of hormones (including hydrocortisone) apparently involves an induction of messenger RNA synthesis, because their action is specifically blocked by actinomycin. Now we have found that when the hydrocortisone induction of enzyme synthesis is blocked by actinomycin, the appearance of the characteristic peaks in the electrophoresis patterns is blocked also. It appears, therefore, that some of these saline-soluble nuclear proteins are involved in some way in the synthesis of messenger RNA.

Others have shown that actinomycin blocks the synthesis of messenger RNA by combining specifically with certain sites in the DNA. We have found that when actinomycin combines with the DNA in isolated nuclei, protein is liberated. Most of this protein shows electrophoretic mobility in the range $+1$ to $-0.8 \text{ cm}^2 \times 10^{-5} \text{ v}^{-1} \text{ sec}^{-1}$; presumably it is combined originally with the DNA in the nucleus, from which it is displaced by the addition of the actinomycin.

Extending our studies with hydrocortisone, we have found that administration of the hormone produces an enlargement of the nuclei in the liver cells, and apparent-

ly a small increase in total nuclear protein. At present, we are attempting to locate this increase more precisely by extracting the isolated nuclei successively with isotonic saline, with saline containing EDTA, with phosphate buffer, and finally with hydrochloric acid to remove the histones. Results to date indicate that administration of hydrocortisone produces a 20 to 25 percent increase in the phosphate-soluble protein and little change in the other fractions. The bulk of the protein in the histone fraction extracted by hydrochloric acid migrates with mobility in the range $+6$ to $+10$ $\text{cm}^2 \times 10^{-5} \text{ v}^{-1} \text{ sec}^{-1}$ and thus is much more basic than the proteins in the saline-soluble fraction.

CONCLUSIONS

The work of the past year has produced additional evidence that changes in the metabolic status of the liver are accompanied by recognizable changes in the quantity and properties of certain nuclear proteins. Work in many laboratories suggests that the principal continuing function of the cell nucleus is expression of the genes; this involves the synthesis of messenger RNA, whose structure embodies the genetic information in the chromosomes (DNA). At present there is a great deal of

interest in this process and in the means whereby it is regulated. Our work of the past year indicates that some of our nuclear proteins probably are involved in some way in this aspect of nuclear function.

REFERENCES

1. Barton, A. D. Soluble proteins from liver cell nuclei. *Z. Zellforsch. Mikroskop. Anat.* **64** (1), 74-82 (1964).
2. Barton, A. D. and E. A. Cerny. Nuclear changes in rat liver cells during thioacetamide administration. Argonne National Laboratory Biological and Medical Research Division Semiannual Report, July through December 1963. ANL-6906, pp. 102-105.
3. Barton, A. D. and K. M. Tracy. Nuclear changes in rat kidney cells after castration and administration of testosterone. Argonne National Laboratory Biological and Medical Research Division Semiannual Report, July through December 1963. ANL-6906, pp. 106-109.
4. Barton, A. D., E. A. Cerny, and K. M. Tracy. Effects of administration of thioacetamide or testosterone on chromosome-associated proteins in rat liver and kidney. *Arch. Biochem. Biophys.* **109**, 36-40 (1965).
5. Barton, A. D. Progress report: Soluble proteins from cell nuclei. Argonne National Laboratory Biological and Medical Research Division Semiannual Report, July through December 1962. ANL-6790, p. 138.
6. Barton, A. D. Progress report: Soluble proteins from cell nuclei. Argonne National Laboratory Biological and Medical Research Division Semiannual Report, January through June 1963. ANL-6823, p. 124.

STABILITY OF THE SECONDARY STRUCTURE OF NUCLEIC ACIDS

Lyle G. Bunville and Sandra J. Schwalbe

PURPOSE AND METHODS

Of the several possible interactions in the bases in DNA that could give rise to the observed stability in aqueous solution, that of hydrogen bonding is most amenable to analysis in terms of changes in stability that result from isotopic exchange. Specific methods that have been used in this investigation are determination of hydrogen-ion binding (determination of protons or deuterons bound as a function of pH) and melting point studies (measurement of fractional changes in absorbance in the ultraviolet as a function of temperature), the solvent in each case being either D_2O or H_2O .

PROGRESS REPORT

Replacement of the solvent H_2O by D_2O has been shown to have no effect on the thermal denaturation of DNA⁽¹⁾ or synthetic polynucleotides⁽²⁾ as judged by changes in optical properties in the ultraviolet or infrared, respectively. Similar results have been obtained for the isothermal, acid-induced denaturation of DNA, at least for that part of the transition comprised of the most stable residues.⁽³⁾ These results have, however, been obtained in solutions of moderately high salt con-

centration (about $0.1M$ NaCl), which has the effect of minimizing small differences in stability that arise from inhomogeneities in base composition, either inter- or intramolecular. Such distributions of stability are reflected in the dispersion of the melting point (variation with wavelength of the median temperature of the melting profile). A detailed analysis, in terms of intermolecular heterogeneity, of this dispersion has been presented by Felsenfeld and Sandeen.⁽⁴⁾

The effect of high salt concentration in minimizing the differences of various features of the denaturation that arise from small differences in stability (either in interactions in base pairs within the helix, or interaction of bases in the coil or with the solvent) are seen when the denaturation in $0.15M$ NaCl is compared to that in $1.5 \times 10^{-3}M$ NaCl, in H_2O . The dispersion of the mean melting temperature, $\Delta t_m = t_m(\lambda) - t_m(259 \text{ m}\mu)$, is shown in Table 1 for a sample of DNA isolated from salmon testis. These results suggest, then, that small perturbations in the helix-coil transition (denaturation) not discernible at high salt concentration might become apparent at low salt concentration. The result in D_2O in $1.5 \times 10^{-3}M$ NaCl is a median temperature, t_m , of

57.68, or an increase in t_m of 1.75°. The dispersion of the melting temperature in D₂O is quite similar to that noted above in H₂O, tentatively indicating that no base, or base pair is preferentially affected by the change of solvent, but that some nonspecific solute-solvent interaction is perturbed, most reasonably the interaction of the phosphate groups of the polynucleotide with sodium ions.

TABLE 1
DISPERSION OF THE MEAN MELTING
TEMPERATURE FOR DNA FROM
SALMON TESTIS

λ , m μ	Δt_m	
	0.15M NaCl	$1.5 \times 10^{-2}M$ NaCl
240	0.11	0.33
250	0.14	0.11
270	— 0.09	0.15
280	0.16	1.03
290	— 0.19	1.28
t_m 259	87.65	55.93

Features of the melting profile other than t_m that are amenable to analysis in terms of perturbation of the stability of the secondary structure are the breadth of the transition and its symmetry. A reasonable fit to the observed data is given by the following:

$$f = \frac{Ke^{-a/T}}{1 + Ke^{-a/T}} \quad (1)$$

where f is the fractional change in absorbance at the temperature T in degrees Kelvin, and K and a are constants evaluated from the data. Although Equation 1 is of the same form as the van't Hoff equation in which K and

a are proportional to the change in entropy and enthalpy, respectively, for a given transformation, no such interpretation is intended, in view of the irreversibility of the denaturation. Empirically, then, a is a function of the breadth of the transition, and K is a function of the breadth and the melting temperature.

In addition to the pronounced lowering of t_m that results from a decrease in salt concentration shown in Table 1, not only does the transition become much broader, but it becomes partially resolved into two components, the best fit of the data requiring two values of a in Equation 1, rather than the single value at high salt. The part of the transition most perturbed by a change in salt concentration is that corresponding to the melting of the most stable residues. Similar results obtain in the case of low salt concentration in D₂O, with the exception of the increase in melting temperature noted earlier.

CONCLUSIONS

It is reasonable to conclude that the helix-coil transition in DNA is affected by isotope exchange, but that solute-solute, solute-solvent interactions are perturbed to such a small extent as to be swamped out at either high salt concentrations or high temperature.

REFERENCES

1. Crespi, H. L. and J. J. Katz. Thermal denaturation of ordinary and fully deuteriated DNA in H₂O and D₂O. *J. Mol. Biol.* 4, 65-68 (1962).
2. Miles, H. A comparison of the thermal dissociation of two polynucleotide helices in H₂O and D₂O. *Biochim. Biophys. Acta* 43, 353-355 (1960).
3. Bunville, L. G. Effect of isotope exchange on the protonation of DNA. Abstracts of the 8th Annual Meeting of the Biophysical Society, WB4, 1964.
4. Felsenfeld, G. and G. Sandeen. The dispersion of the hyperchromic effect in thermally induced transitions of nucleic acids. *J. Mol. Biol.* 5, 587-610 (1962).

ATTEMPT AT BREEDING A LOW BLOOD CATALASE STRAIN OF MOUSE

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

PURPOSE AND METHODS

We have earlier indicated our interest in obtaining a strain of mouse essentially devoid of blood catalase ("acatalatic").⁽¹⁾ One possible, though unlikely, way to achieve this seemed to be the empirical breeding of low catalase strains, with continued crossbreeding of low catalase progeny. The first step in this project was described in an earlier report,⁽²⁾ in which the results of blood, liver, and whole body catalase assays were described for 19 different inbred strains.

PROGRESS REPORT

Strains finally selected for initiation of this project, together with blood catalase levels expressed as perborate units per ml (PU/ml)⁽³⁾ are as follows:

C 57 BL/J	121 PU/ml
MA/J	74
C 58/J	119
ST/J	107

These four strains were "double-crossed" by stand-

ard techniques,* and the progeny of the cross were then assayed and mated on an empirical basis, discarding high catalase animals and mating low catalase ones.

In brief, this process was found ineffective. After approximately 19 months of such breeding attempts, an animal was obtained with a blood catalase level of 58 PU/ml, the lowest observed in this experiment. This animal, however, proved to be sterile and so was the end of his line. A repeat mating of his parents produced nothing comparable. Since only a half dozen or so mice

* We wish to extend our warmest thanks to Dr. John B. Storer, Jackson Memorial Laboratory, for gifts of mice and for initiating the "double-crossed" stock for us.

EFFECT OF COMPOUNDS RELATED TO AMINOTRIAZOLE ON BLOOD AND LIVER CATALASE

Robert N. Feinstein and John E. Seaholm

PURPOSE AND METHODS

We recently published a description of the effects of some 64 compounds related to aminotriazole on catalase *in vivo* and *in vitro*.⁽¹⁾ Although our chief source of new compounds is no longer available to us, we have continued to search the literature, chemical manufacturers' production lists, etc., for additional compounds and have tested these. Testing has been limited to effects on blood and liver catalase *in vivo* unless significant inhibitory effects are observed, in which case it is planned to analyze the inhibitory effects more closely by *in vitro* techniques.

PROGRESS REPORT

The following compounds have been tested since the publication of the earlier paper:

(a) Gift of the Geigy Research Laboratories, Ardsley, New York*

Atrazine
Simazine
Propazine
Prometone

* We wish to thank Dr. Murray Weiner of the Geigy Research Laboratories for generous supplies of these drugs.

SEARCH FOR AN ACATALASEMIC MOUSE. II.

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

PURPOSE AND METHODS

In a recent report, we described the beginning of a large-scale mouse screening test designed to screen out a mutant animal low in blood catalase.⁽¹⁾ Mice tested were the progeny of irradiated sires,* and they were

had been produced with blood catalase levels appreciably lower than that of the initial MA/J mice, the effort was discontinued at this point.

REFERENCES

1. Feinstein, R. N. and J. Dainko. Use of 3-amino-1,2,4-triazole to reduce body catalase activity in cancer studies. *Cancer Res.* **19**, 612-617 (1959).
2. Feinstein, R. N. and M. Coulon. Catalase activity in various inbred mouse strains. Argonne National Laboratory Biological and Medical Research Division Semiannual Report, January through June 1962. ANL-6723, pp. 208-210.
3. Feinstein, R. N. Perborate as substrate in a new assay of catalase. *J. Biol. Chem.* **180**, 1197-1202 (1949).

Prometryn

Trietazine

Chlorazine

These are all variously substituted triazines.

(b) Purchased from Nutritional Biochemical Corp., Cleveland, Ohio

Glyoxal bis guanyl hydrazone

Methyl glyoxal bis guanyl hydrazone

(c) Purchased from the Aldrich Chemical Company, Milwaukee, Wisconsin

3-amino carbazole

3-amino pyrazole

3-pyrrolindole

7-azaindole

4,5-dihydro-6-methyl-3(2H)-pyridazinone

N(1H-1,2,4-triazol-3-yl)-maleimide

2-amino-4-anilino-6-chloromethyl-s-triazine

All of the above compounds were totally ineffective in inhibiting either blood or liver catalase *in vivo* when administered at the highest practicable (i.e., nonlethal) dose.

REFERENCE

1. Feinstein, R. N., J. E. Seaholm and L. B. Ballonoff. Effect of compounds related to aminotriazole on catalase *in vivo* and *in vitro*. *Enzymologia* **27**, 30-40 (1964).

screened by a recently described, rapid, semiquantitative assay for blood catalase.⁽²⁾

* We wish to acknowledge our deep indebtedness to Dr. W. L. Russell, Oak Ridge National Laboratory, for the gift of the mice and for helpful discussion.

PROGRESS REPORT

The screening portion of this project is now completed. Altogether, 12,306 mice were examined on a semiquantitative basis, and of these, 56 animals were designated by the screening test as having a subnormal blood catalase level [below approximately 115 perborate units (PU) per ml, as contrasted with a normal population showing a mean blood catalase of 149 PU/ml]. The 56 animals were then assayed quantitatively for blood catalase,⁽³⁾ and 42 of the selected mice were found to have blood catalase of 115 PU/ml or lower. These 42 mice were then tested for catalase aberrations by standard genetic techniques (mating with normal animals, then backcrossing low catalase progeny with the suspect parent). In a few instances, when the original suspect was near 115 PU/ml, and the F₁ generation seemed to show no instances of low blood catalase, backcrosses were not carried out, and it was considered that the original suspect animal was actually normal.

Of the 42 animals thus tested genetically, all but seven have by this time been eliminated from further consideration. The remaining seven animals may be briefly described as follows:

a) One mouse, female 2007, blood catalase 40 PU/ml, is definitely a catalase mutant. She is by now the progenitor of numerous progeny even unto the fourth generation. It has been established that the heterozygote range of blood catalase is ca. 65–80 PU/ml, and the homozygote range, ca. 0.5–6.5 PU/ml. If homozygotes or heterozygotes are mated with heterozygotes or with normal mice, the proportion of heterozygote and of homozygote progeny is lower than expected, and the proportion of normal progeny is higher. Homozygote-homozygote matings, however, produce adequate litters, all members of which breed true. A colony of essentially acatalasemic mice, genetically defective at one locus only, has thus been established.

b) One mouse, male 2003, blood catalase 92 PU/ml, has also been established to be a genetic aberrant with respect to blood catalase. The phenotype in this in-

stance, however, is much more variable than that of a) above. The heterozygote apparently expresses itself anywhere from 90 PU/ml to as high as 135 PU/ml (which is ordinarily considered well within the normal range). The homozygote evidently appears as a blood catalase level of ca. 30–55 PU/ml. We are not yet able to say with assurance that these homozygotes will breed true, though we expect this to be the case. Nor are we yet able to state whether this mutant is allelic with, or independent of, the one described in a) above. The experiments necessary to establish these points are under way.

c) Two other mice, male 2043 and male 2044, with blood catalase levels of 86 and 83 PU/ml, respectively, are also in all probability genetically abnormal with respect to blood catalase. The picture here, however, is complicated by the concomitant occurrence of an anemia. The interesting possibility exists that these two mutants may reflect malsynthesis of the heme portion of the catalase (which would thus probably also affect hemoglobin, the cytochromes, etc.), whereas the first two listed mutants are malsynthesizing the apocenzyme, the protein moiety, of the catalase. The unfortunate possibility must also be considered that these heme aberrants, if such they be, may not be viable in the homozygote condition because of the variety and seriousness of the defects generated.

d) Lastly, three other suspect animals, males 2013, 2033, and 2041, with blood catalase of 102, 93, and 110 PU/ml, respectively, are not yet far enough along in their genetic analysis for any statements to be made.

REFERENCES

1. Feinstein, R. N., J. E. Seaholm and J. B. Howard. Search for an acatalasemic mouse. Argonne National Laboratory Biological and Medical Research Division Semiannual Report, July through December 1963. ANL-6906, pp. 75–77.
2. Feinstein, R. N., J. B. Howard, L. B. Ballonoff and J. E. Seaholm. A rapid blood catalase screening technique adjustable to any level of activity. *Anal. Biochem.* **8**, 277–281 (1964).
3. Feinstein, R. N. Perborate as substrate in a new assay of catalase. *J. Biol. Chem.* **180**, 1197–1202 (1949).

SEARCH FOR A MOUSE GENETICALLY DEFICIENT IN BLOOD GLUTATHIONE PEROXIDASE

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

PURPOSE AND METHODS

Elsewhere in this report we have described our search for a mutant mouse genetically subnormal in blood catalase activity. The basic reason for desiring such a mouse is not for the catalase lack per se, but rather to

have a mouse essentially incapable of destroying H₂O₂. [The rationale is more fully described in an earlier paper.⁽¹⁾] Cohen and Hochstein⁽²⁾ have recently presented evidence suggesting that glutathione peroxidase (GSH-POX), an enzyme found in many tissues but most prom-

inently in the erythrocytes, may be of even greater importance than catalase in the removal of H_2O_2 formed by irradiation, flavoprotein enzymes, etc. Thus we decided that, while we were screening a population of mice, progeny of irradiated sires, for low blood catalase, we would also examine these mice for a mutant low in blood glutathione peroxidase.

No method was available in the literature to serve as a rapid, semiquantitative screening test for the enzyme, and the only published quantitative assay methods⁽³⁾ did not appear to us to be suitable for the fairly large number of animals we expected to be testing on a quantitative basis. It was, therefore, necessary for us to devise both a rapid, semiquantitative assay suitable for very large numbers of animals, and also a more quantitative assay that could be used routinely on smaller, but still appreciable, numbers of mice. These are briefly described below.

Semiquantitative Screening Assay

Blood was drawn from the orbital sinus.⁽⁴⁾ Since these assays were done concurrently with blood catalase screening, two sets of blood-collecting tubes were prepared, one marked at 35 mm (70 microliters) for catalase assay, the other marked at 25 mm (50 microliters) for GSH-POX assay. While holding a given animal, first one tube and then the other was properly filled. This obviated the need for transfer of aliquots, with the attendant consumption of time and use of extra glassware, etc. The tube lengths noted were those found by preliminary empirical test to provide sufficient enzyme to carry out the reaction if the blood were normal, but to provide insufficient enzyme to carry out the reaction if the blood activity were below the level of activity of low normal. Particularly in the case of the GSH-POX, for which there was then no suitable quantitative assay, the determination of this value was a somewhat arbitrary decision. However, it was found that a series of presumably normal blood samples, if tested at 50 microliters, satisfactorily destroyed the H_2O_2 , while the same bloods tested in appreciably lesser volumes did not.

The blood from the GSH-POX tube was introduced into a 50-ml Erlenmeyer flask containing 5 ml of phosphate buffer ($M/15$, pH 7.4) and 10 ml of a solution of EDTA ($4 \times 10^{-4} M$), sodium azide ($9 \times 10^{-3} M$), and reduced glutathione (GSH) ($1.6 \times 10^{-3} M$). After the blood had laked, 5 ml of H_2O_2 solution ($9 \times 10^{-4} M$) were added, and the whole was mixed by swirling. After about ten minutes—the time is not critical—the solutions were examined. If the blood were normal with respect to GSH-POX, the enzyme destroyed the H_2O_2 by oxidation of GSH, and the mixture retained its normal,

light pink, hemoglobin color. On the other hand, if the blood were deficient in GSH-POX, the H_2O_2 was not destroyed rapidly enough, and residual H_2O_2 oxidized the hemoglobin to brown shades of mixtures of methemoglobin, choleglobin, and perhaps other forms.

Quantitative Assay

The general principle of this method, which is still not completely quantitative, is as follows. Blood is laked in a solution containing EDTA (to minimize the heavy-metal catalyzed nonenzymic oxidation of GSH), sodium azide (to inhibit catalase activity), and GSH. The reaction, buffered with phosphate at pH 7.4, is started by the addition of H_2O_2 and halted by the addition of HPO_3 . The precipitated protein is centrifuged down, and to an aliquot of the filtrate is added a buffered (pH 7.4) solution of 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB).⁽⁵⁾ The resulting yellow color, a measure of the GSH content, is read at 412 m μ . The initial GSH content of the mixture is obtained by preparing, in each case, another mixture identical with the first except that the HPO_3 is added first, thus preventing any possible enzymatic oxidation of the GSH. With each set of test mixtures, one blood-free pair is included to measure the nonenzymatic decrease in GSH, which is then subtracted from the total decrease. Final concentrations of the various components in the incubating mixture (not including the deproteinizing HPO_3) are: EDTA $2.6 \times 10^{-4} M$, NaN_3 $4.6 \times 10^{-3} M$, GSH $6.5 \times 10^{-4} M$, and H_2O_2 $4.4 \times 10^{-3} M$. The total volume of the mixture is 10 ml, and it is deproteinized with 3 ml of 3% HPO_3 .

The GSH-POX is evidently highly unstable under these circumstances. Despite the presence of adequate GSH and H_2O_2 , the amount of GSH disappearing during the second minute of the reaction is 50% or less of that disappearing during the first minute. For this reason, we use only two time points—zero and one minute. Even under these conditions, the reaction is not perfectly linear with respect to enzyme concentration, i.e., blood volume. One ml of 1:25 blood causes 1.7–1.8 times as much GSH oxidation as 1 ml of 1:50 blood. This assay method is thus by no means ideal, but it is rapid, reproducible, and sufficiently quantitative to permit the clear recognition of abnormal blood samples, which is our need.

PROGRESS REPORT

In all, 3358 mice were screened by the semiquantitative test described. Of these, 10 were set aside as being possibly subnormal in GSH-POX activity. These ten have been examined by the more nearly quantitative assay described above, and two appear low, though still

within the normal range. At this point it appears unlikely, though still possible, that we have a GSH-POX mutant.

REFERENCES

1. Feinstein, R. N. and J. Dainko. Use of 3-amino-1,2,4-triazole to reduce body catalase activity in cancer studies. *Cancer Res.* **19**, 612-617 (1959).

SEARCH FOR INBORN ERRORS OF METABOLISM IN MICE BY URINE TESTING

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

PURPOSE AND METHODS

Elsewhere in this report we have described a screening search for mice with low blood catalase and with low blood glutathione peroxidase. The mice tested were the progeny of irradiated sires and, hence, were expected to exhibit a higher than normal incidence of mutation. Because of the unusual nature of this population, we wished to utilize it maximally. Therefore, after the mice were screened for blood catalase and for blood glutathione peroxidase, they were transferred in groups of 15 to stainless steel cages bottomed by stainless steel pans with no sawdust or other bedding. Water was provided *ad lib.*, but food was withheld in order to prevent contamination of the urine. After 15-20 hours, the urine pool was collected, filtered free of feces through glass wool, and was then tested with a battery of dipstick and drop tests. The following tests were employed:

- (a) Ketone bodies (Ames Company Ketostix®)
- (b) Phenylpyruvic acid (Ames Company Phenistix®)
- (c) Glucose (specific) and occult blood (Ames Company Hema-combistix™)
- (d) Protein (Ames Company Albutest®)
- (e) Sugar (general reducing substances) (Ames Company Clinitest®)
- (f) Cystine and homocystine (Ames Company Acetest®, modified according to Ames Company instructions)
- (g) Bilirubin (Ames Company Ictotest®)
- (h) Acid mucopolysaccharides (toluidine blue, modified slightly from Berry and Spinanger⁽¹⁾)

EFFECTS OF PUROMYCIN ON ERYTHROCYTE CATALASE

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

PURPOSE AND METHODS

We recently demonstrated that puromycin, administered intraperitoneally eight times during the course of four days, brought about a 56% reduction in liver catalase by the fifth day, although the erythrocyte

2. Cohen, G. and P. Hochstein. Glutathione peroxidase: the primary agent for the elimination of hydrogen peroxide in erythrocytes. *Biochemistry* **2**, 1420-1428 (1963).
3. Mills, G. C. The purification and properties of glutathione peroxidase of erythrocytes. *J. Biol. Chem.* **234**, 502-506 (1959).
4. Riley, V. Adaptation of orbital bleeding technique to rapid serial blood studies. *Proc. Soc. Exptl. Biol. Med.* **104**, 751-754 (1960).
5. Ellman, G. L. Tissue sulfhydryl groups. *Arch. Biochem. Biophys.* **82**, 70-77 (1959).

- (i) Tyrosine (Millon's Reagent, as suggested by Berry⁽²⁾)
- (j) Proline (isatin reaction, as suggested by Berry⁽²⁾)
- (k) Copper (orthotolidine-NH₄CNS, as suggested by Berry⁽²⁾)

PROGRESS REPORT

Over 7200 mice were tested with most of these urine tests. On several occasions ketone bodies were found, but these disappeared when the animals were fed. Positive tests for occult blood were very frequently found. This was traced to the feces, which gave very strongly positive "occult blood" tests. An attempt to sterilize the gut by incorporation of neomycin in the drinking water failed to remove the positive "occult blood" tests from the feces, and the matter was pursued no further.

CONCLUSIONS

The mice examined, progeny of irradiated sires, showed no evident biochemical abnormality in the urine, as tested by a battery of eleven tests. This is perhaps not an unexpected finding, since the animals examined would probably be only heterozygotes, if at all abnormal genetically, and heterozygotes are not commonly known to excrete abnormally.

REFERENCES

1. Berry, H. K. and J. Spinanger. A paper spot test useful in study of Hurler's syndrome. *J. Lab. Clin. Med.* **55**, 136-138 (1960).
2. Berry, H. K. Detection of metabolic disorders among mentally retarded children by means of paper spot tests. *Am. J. Mental Deficiency* **66**, 555-560 (1962).

catalase was essentially unaffected.⁽¹⁾ Since we are primarily interested in techniques for reducing blood catalase,⁽²⁾ we have now performed a longer experiment, in which it was hoped the puromycin would inhibit catalase synthesis in the bone marrow, ultimately putting a

"pulse" of catalase-free erythrocytes into the peripheral circulation.

Puromycin was injected intraperitoneally at 100 mg/kg, twice on day one, once on day two. Blood was withdrawn by the orbital sinus technique⁽³⁾ and assayed by the perborate method.⁽⁴⁾

PROGRESS REPORT

Blood catalase was assayed twice weekly for eleven weeks. At no time was a significant decrease in activity noted.

TOXICOLOGICAL STUDIES OF TRITIATED PRECURSORS

Walter E. Kisieleski

PURPOSE AND METHODS

In recent years, radioactive thymidine, cytidine and leucine have been extensively used as precursors, respectively, of DNA, RNA, and proteins. For some time there has been concern as to possible radiotoxicity of these materials, both with respect to introducing an artifact into experimental results and with respect to the safety of laboratory workers handling the material. Concern for possible toxic effects of tritiated thymidine was voiced early, for it was recognized that because of its incorporation into the very substance of nuclear material a rather small amount of energy from the 18-keV maximum beta emission might produce effects of some consequence. We felt that effects of tritiated thymidine exposure might be more closely related to uptake than to administered dose. Thus, we undertook to investigate the uptake and retention of tritiated thymidine, cytidine, and leucine in the laboratory mouse and examine possible parameters for toxicity measurements.⁽¹⁾ Experiments were also designed to test the possibility that the route of administration may have a measurable influence on the uptake of tritiated compounds by mouse tissues *in vivo*.

PROGRESS REPORT

Uptake and retention of tritiated thymidine activity were found to be only partially related to total dose given at a single time. Dividing of doses into serial parts increased uptake and retention, whereas increasing the specific activity of tritiated thymidine used or increasing the age of the animal decreased uptake and retention. Further experiments showed that the route of administration has a measurable effect on the uptake of tritiated compounds such as thymidine and cytidine and that this effect is detectable by both radiochemical and autoradiographic methods.

REFERENCES

1. Feinstein, R. N., J. B. Howard and J. E. Seaholm. Effect of puromycin on blood and liver catalase. Argonne National Laboratory Biological and Medical Research Division Semi-annual Report, July through December 1963. ANL-6906, pp. 70-72.
2. Feinstein, R. N. and J. Dainko. Use of 3-amino-1,2,4-triazole to reduce body catalase activity in cancer studies. *Cancer Res.* **19**, 612-617 (1959).
3. Riley, V. Adaptation of orbital bleeding technique to rapid serial blood studies. *Proc. Soc. Exptl. Biol. Med.* **104**, 751-754 (1960).
4. Feinstein, R. N. Perborate as substrate in a new assay of catalase. *J. Biol. Chem.* **180**, 1197-1202 (1949).

In the intact mouse thymidine was best utilized when injected subcutaneously and cytidine when injected intravenously. The fractional distribution of the radioactivity incorporated into DNA after administration of thymidine, was substantially similar for the intravenous and the subcutaneous route, while the intraperitoneal injection caused an increased amount of radioactivity to accumulate in the gastrointestinal tract and the spleen. An intraperitoneal injection of thymidine into mice bearing intraperitoneal growths of Ehrlich ascites tumor cells also resulted in an increased labeling of tumor cells, when compared to a subcutaneous injection. However, the percentage of labeled cells was not affected by the route of injection.⁽²⁾

The uptake of cytidine by muscle cells belonging to different organs was markedly influenced by the route of administration. Cells of organs directly exposed to an intraperitoneal injection were more intensely labeled after intraperitoneal than after intravenous injection of cytidine. The opposite was true of cells of organs not directly exposed to an intraperitoneal injection. The results with leucine were inconclusive and differences due to the route of administration are not statistically significant.

Variations in the uptake of a radioactive precursor due to the route of administration are of limited importance when the same organ or tissue is investigated in respect to variations in time or treatment. However, in comparing the rates of DNA, RNA, or protein synthesis in different tissues, one should remember that the uptake of tritiated precursors and the intensity of the autoradiographic label are differently affected in different organs by the route of administration.

CONCLUSION

It is suggested that toxicity of tritiated thymidine and possibly other tritiated compounds is best related to

uptake and retention rather than to administered dose, and that factors influencing uptake and retention may play a more important role in expressed radiotoxicity than has been realized.^(3, 4)

REFERENCES

1. Samuels, L. D. and W. E. Kisieleski. Toxicological studies of tritiated thymidine. *Radiation Res.* **18**, 620-632 (1963).
2. Baserga, R. and W. E. Kisieleski. Reutilization of labeled DNA by Ehrlich ascites tumor cells. *Arch. Ital. Path. Clin. Tumori* **6**, 1-6 (1963).
3. Samuels, L. D., W. E. Kisieleski and R. Baserga. Tritated thymidine toxicity in mammalian systems. *Atompraxis* **10**, 144-148 (1964).
4. Kisieleski, W. E., L. D. Samuels and P. C. Hiley. Dose effect measurements of radiation following administration of tritiated thymidine. *Nature* **202**, 458-459 (1964).

STEROL METABOLISM

Peter D. Klein, John C. Knight, and Patricia A. Szczepanik

PURPOSE AND METHODS

The object of this study is to clarify some of the details of the transformation of lanosterol to cholesterol in the liver and to demonstrate which of the many possible theoretical pathways constitutes the route actually followed in the normal animal. The individual steps involved consisted of:

- 1) the removal of the three methyl groups;
- 2) the saturation of the side chain double bond; and
- 3) the transfer of the nuclear 8(9) double bond to the 5(6) position.

In spite of the large amount of published work on the subject, few of the possible intermediates have been isolated, and there is some conflict of opinion concerning the order in which certain steps occur. Much of the work has been carried out with drug-treated animals in the hope that certain enzyme systems would be blocked, causing the accumulation in the cells of otherwise short-lived intermediates, but whether or not these results can be taken as applicable to normal animals is open to question.

The transformation takes place very quickly *in vivo*, so that within 15 minutes of the injection of tritium- or carbon-14-labeled mevalonic acid into a rat, lanosterol has been synthesized and almost completely converted into cholesterol. If the animal is killed within a shorter time period, however, then a significant proportion of the activity (though a very small percentage of the total mass) is found to reside in sterols other than cholesterol. The presence of these sterols is only detectable by virtue of the radioactivity incorporated into them, and attempts at structure elucidation are thus hindered by the unavailability of spectra, physical constants, analytical data, or any of the other tools usually applied to such problems.

The ready availability of instruments which can provide a sensitive and quantitative measure of radioactivity means that full use can be made of one remaining characteristic of a molecule which can be measured with less than a microgram of material, namely the chromato-

graphic mobility on various adsorbants in different solvent systems. In order to have a quick and reproducible means of identifying the sterols likely to be present in a mixture extracted from a natural source such as rat liver, a large number of fully and partially methylated sterols, with double bonds in various positions, have been synthesized. Their relative elution volumes (REV), expressed on the basis of cholesterol acetate = 1.00, have been determined on a column of code 12 silica gel which has been carefully equilibrated under standard conditions, and the results plotted to show the dependence of mobility on structural detail (see Figure 1).

The relative elution volume of any sterol which accompanies cholesterol can now be determined on this scale, and if found to be coincident with that of one of the synthetically prepared sterols, then the identity of the two compounds can be established by cochromatography of the naturally occurring compound and the synthetic material, each labeled with a different isotope. Subtle differences between two otherwise similar molecules can be detected by means of the resultant slope in a plot of the isotope ratio in each fraction. A constant isotope ratio across a peak demonstrates complete identity.

PROGRESS REPORT

It has been found that a sterol with an REV of 1.54 is consistently present in preparations of rat liver sterols. This product accounts for about 20% of the total radioactivity though only a minute percentage of the mass, and because of this it seems probable that it is a genuine intermediate. Thus, elucidation of its structure would shed some light on the biosynthetic route to cholesterol, and some progress has already been made to this end. Its REV, falling between those of cholest-7-ene-3 β -ol acetate and cholesta-7,9(11)-diene-3 β -ol acetate (see Figure 1), does not correspond to that of any sterol with a biosynthetically plausible structure, but saponification and acetylation yields a product that has been identified as cholest-7-ene-3 β -ol acetate, and this

has been confirmed by reduction to cholestan- β -ol acetate by hydrogenation over platinum.

Reduction with lithium aluminum hydride gives a product which has been resolved into three components, one of which has been shown to consist mainly of cetyl alcohol, revealing the presence of palmitic acid in the original molecule. The cetyl alcohol peak obtained from a preparation of the sterols from eight rat livers has been analyzed with the aid of a computer program, and from this it has been deduced that the cetyl alcohol is accompanied by myristyl and stearyl alcohols, each in a concentration of about 15%. On the basis of the specific activity of the acetic anhydride used to acetylate the alcohol mixture before chromatography, it has been calculated that the total amount of fatty alcohols obtained was of the order of 3 micrograms. Further work on this problem is being carried out, the evidence at present favoring a dihydroxycholestane type of structure.

The removal of the three methyl groups of lanosterol has been shown to require oxygen, and quantitative experiments have demonstrated that for each molecule of cholesterol produced, three molecules of carbon dioxide are liberated. This makes it likely that the methyls are

removed by oxidation to carboxylic acid groups followed by decarboxylation. Bloch has isolated 14-desmethyl lanosterol from rats killed almost immediately after injection, and so has postulated that the 14-methyl group is the first to go, and that the decarboxylation is aided by the double bond in the 8(9) position. However, the generally accepted mechanism for the decarboxylation of γ,δ -unsaturated acids would require a simultaneous shift of the 8(9) double bond to the 8(14) position, and it has not been explained why this does not occur. It is proposed to investigate this reaction further if a suitable photochemical rearrangement can be worked out to convert the 14-methyl into a 14-carboxyl group.

The elimination of a carboxyl group from the 4-position would be greatly facilitated if the 3β -hydroxyl were converted to a keto group since β -keto acids decarboxylate spontaneously, and a study of the participation of ketones in the biosynthetic sequence has, therefore, been initiated. If the 24(25) double bond in lanosterol is directly hydrogenated with tritium gas and Raney nickel catalyst, a high degree of labeling can be obtained with the label in a stable position remote from the

FIG. 1.—Dependence of mobility of sterols on structure

sites of the enzymatic reactions involved. The resultant radioactive dihydrolanosterol is readily converted by the Oppenauer procedure to the corresponding 3-ketone, and the behavior of this compound on incubation with rat liver homogenates under a variety of conditions has been studied in detail. An acidic product that was consistently obtained has been shown to result from a non-enzymatic oxidation catalyzed by metal ions, and this is under further study. Apart from this, utilization of the ketone is very slow, and the only sterol (obtained in about 20% yield) produced has been shown to be cholesterol. This was identified by dual label chromatography, which showed the REV to be 0.9991. Incubation of dihydrolanosterol gave a variety of sterol

products which have not yet been identified, and work on this aspect of the problem is still proceeding.

This procedure of labeling a compound by direct tritiation of a double bond has many potential uses which we hope to explore in the near future. For instance, it has been frequently claimed that 5,7-dienes and 5,7,24-trienes are important intermediates, but such compounds tend to polymerize on the silica surface and so cannot readily be chromatographed. Treatment with Raney nickel and tritium gas would not only convert them to stable 7-enes but would also introduce a specific label, enabling minute quantities of such compounds to be investigated in the presence of much larger amounts of other sterols.

THE USE OF DUAL TRACER TECHNIQUES IN CHROMATOGRAPHIC STUDIES

Peter D. Klein and Patricia A. Szczepanik

PURPOSE AND METHODS

This project seeks new analytical methods and conceptual attacks for which dual tracer techniques have a particular suitability.

The advent of automatic liquid scintillation spectrometers has made possible a great expansion in the design and number of experiments using two forms of radioactivity. The commonest pair of this instance is H^3 and C^{14} , though other beta-emitter pairs and beta-gamma pairs can be used. In essence, the use of two forms of radioactivity permits an internal standardization to be achieved which often permits an experimental comparison which is easier, more convenient, or more revealing. We are particularly concerned in this project with applications to chromatographic analysis and studies of the chromatographic process.

When a substance is placed on a chromatographic column and an appropriate solvent is applied to the top of the column, the substance will migrate down the column and emerge in the column eluate. For a given column and solvent, the rate at which a substance migrates is a function of its structural groups and their location. Under appropriately standardized conditions, this migration rate or relative elution volume (REV) is characteristic of a given structure and can be used to identify an unknown compound. In the class of compounds of particular interest to us, the sterols, two complications arise: first the amounts of material are often so small as to be visible only by virtue of the radioactivity introduced into them, and second, alternative structural combinations may result in relative elution volumes which differ by only a few per cent. A direct comparison of an unknown compound with the known (suspected) structure is possible when one is labeled with

H^3 (by biosynthetic means or by derivative labeling in the conversion to the acetate with H^3 -acetic anhydride) and the other is labeled with C^{14} . Cochromatography of the two compounds permits an assessment of the degree of identity by means of the isotope ratio, H^3/C^{14} . If complete identity exists, the ratio remains constant across the peak from leading to trailing edge. If a difference is present in the migration rates, a rising or falling ratio will be readily evident.

To permit such identifications to be carried out in a routine manner, two types of facilities are required: those concerned with data transcription and computation, and those concerned with mathematical analysis and projection. The former is provided by a direct link between the spectrometer and a key punch which permits transcription onto punched cards and by a series of computational programs developed by the Mathematical Section of the Biology Division. The mathematical analysis of the relationship between the two chromatographic peaks, as displayed by the dual tracer technique, has been carried out, aided in considerable part by a computer program which permits assembly and display of synthetic chromatograms. This program is based on the demonstrable principle that a chromatographic peak is best described in terms of the mean (M), area (n) and dispersion (σ) of a Gaussian curve. The program will assemble a chromatogram from a total of eight components (four H^3 and four C^{14}) when these are specified by the variables listed.

PROGRESS REPORT

Preliminary or significant progress has been made in the areas of 1) compound identification, 2) purity-homogeneity criteria and 3) analysis of complex mix-

tures without total resolution of the components. Application of the procedures described above, for instance, to standard reference materials such as cholesterol acetate- H^3 and cholesterol acetate- C^{14} has revealed that the methods are sensitive enough to detect mobility differences between the H^3 - and C^{14} -labeled forms, i.e., effects at the isotopic level. This difference, which may be minimized by appropriate intramolecular positioning of the two isotopes, can be detected with these mathematical analyses at astonishingly low levels; to date, mobility differences as low as 12 parts in 100,000 have been measured with accuracies of 5 parts in 100,000.

The presence of these effects always raises the bugaboo of possible radiochemical contamination in the chromatographic peaks, and it became important to determine what effect the presence of contaminants would have on the isotope ratio and on the characteristics of the chromatographic peak. The synthetic chromatogram computer program was used to generate a variety of "contaminated" chromatograms and these were then analyzed for the resulting changes in mean, area and dispersion, as well as for the changes in isotope ratio of successive fractions across the peak. The resulting equations demonstrated that every introduction of a contaminant results in both a displacement of the mean as well as an increase in the dispersion of peak broadness. If one compares an experimental sample with a pure reference sample on the same column (as can be done with the dual tracer technique), one can determine the degree of displacement and peak broadening present and, thereby, determine the likelihood of contamination. Analysis in this manner has shown that radiochemical contamination is not responsible for the displacement in mean, or relative elution volume, observed in these columns since it would require that the contaminant amount to more than half of the total sample, a requirement which we regard as self-eliminating.

The recognition of the relationship between peak dispersion and the presence of contamination has opened the door to a new extension in the analysis of complex mixtures. If two or three components migrate within closely similar elution volumes (i.e., within 10%), they form a single peak whose dispersion is considerably greater than that of the individual peaks. If one knew the dispersion of any one component and its approximate area, one could construct a synthetic peak of equal size and shape which could be subtracted from the

enveloping peak to reveal the residual components. The use of dual label techniques and the previously described computer programs are ideally suited for this purpose; when a pure reference standard is chromatographed with such a mixture, it provides both a measure of the dispersion of the pure component and an approximate estimate of its concentration in the mixture. The latter is obtained by comparing the isotope ratio in the center of the peak with the isotope ratio of the reference to the mixture as a whole. This indirect isotope dilution analysis, together with the dispersion of the reference peak permits a computer generated synthetic peak to be subtracted from the experimental chromatogram. Appropriate programs then permit measurement of the residual peaks for relative elution volume, dispersion and area. The "exactness of fit" may be examined by recombining the derived peak parameters into an idealized envelope peak and then comparing this peak with the original chromatogram.

This technique has been applied to several experimental mixtures, notably a mixture of aliphatic alcohols obtained from a biological product in sterol metabolism by reductive cleavage of an ester. These alcohols were characterized, according to chain length and concentration in a total sample of less than four micrograms. This technique has also provided evidence for a previously unsuspected intermediate in sterol metabolism, 4,4-dimethyl- Δ^7 -cholesten- 3β -ol. This compound, which migrates at 0.955 relative to cholesterol at 1.000 (when both are present as the acetates), is present in concentrations of less than 2% and is visible only at early time periods after biosynthetic labeling with mevalonic acid- $5-H^3$. It was detected by chromatographing the cholesterol peak obtained at such early time periods with a reference marker of cholesterol acetate- C^{14} . Subtraction of the cholesterol peak in the manner described revealed a component amounting to 15% of the total activity whose migration rate was 0.948 that of cholesterol, an excellent agreement in migration properties with those of the known compound.

CONCLUSIONS

Dual tracer techniques offer a variety of options in analytical procedures and the extent of their utilization, now that equipment and computational procedures are available, is limited only by the ingenuity of the investigator.

BIOCHEMISTRY AND ION BINDING OF CONNECTIVE TISSUE

Arthur Lindenbaum, Abbas O. Jibril, and William M. Westfall

PURPOSE AND METHODS

This study is designed to investigate the biochemical basis for the transformation of cartilage to bone. The over-all objectives are to increase our understanding of normal and abnormal physiological processes in bone, and to develop new concepts and new techniques for the treatment of radioelement poisoning. The approach includes: 1) characterization of components of the ossifying cartilage and bone, particularly the mucopolysaccharide-containing components as they exist *in situ*; and 2) study of their interactions with other substances such as calcium and the polyvalent radioelements taken up by bone.

Initial efforts are directed toward isolation, characterization and quantitation of undegraded components of the organic matrix of cartilage and bone at various stages during calcification and ossification. Of particular interest are the properties of complexes of mucoproteins, lipo- and phosphoproteins, and of associated compounds as they may be isolated and characterized. We are also looking for changes in the physical and chemical properties of the undegraded organic and inorganic components of ossifying cartilage to determine their possible involvement in the uptake of calcium and other substances.

Techniques for isolation and characterization of these substances include ion exchange, polarimetry, spectrophotometry, ultrasonication, chromatography, electrophoresis, and ultracentrifugation. Measurements of the affinity of cartilage and its isolated components for polyvalent cations such as calcium are performed by a variety of techniques (e.g., ion exchange, equilibrium dialysis, titration) to provide relative or absolute binding constants.

A great obstacle to understanding the role of connective tissue components in calcification and ossification is the dearth of useful and reliable methodology. Therefore, the development of new or better methods for characterizing the organic components of cartilage is a continuing secondary objective in this work.

PROGRESS REPORT

The zones in calf scapula designated as "resting" and "transforming" cartilage have been examined with respect to the amino acid content of neutral water extracts of homogenized cartilage, which contain the bulk of the mucoproteins. No significant differences in either kinds or amounts of amino acids have yet been found.

A continuous-recording automatic burette has been constructed and found useful not only for the analysis of

heparin⁽¹⁾ but for the determination of carboxyl and sulfate content of other mucopolysaccharides and of mucoprotein extracts of scapular cartilage. As was anticipated from preliminary work, the sensitivity of the sulfate determination has been increased by the use of solvents of lower dielectric constant (e.g., acetone-water, dioxane-water). These results are being prepared for publication.

Previously, the only gross chemical difference detected between the resting and transforming zones of scapular cartilage was an increase in the calcium content of the latter zone. Sialic acid, which had not previously been found in the lyophilized neutral aqueous mucoprotein extracts or in the isolated mucopolysaccharides of calf scapula, has now been determined in the neutral, alkaline (0.1 *N* NaOH), and acid (0.1 *N* HCl) aqueous extracts of cartilage fractions after removal of lipids with 2:1 chloroform-methanol. Between 0.05 and 4 micromoles sialic acid have been found per gram of fresh cartilage, with the highest concentrations being found in the alkaline extracts. For each type of extraction there was a significant (2- to 4-fold) increase in the sialic acid content of extracts of transforming cartilage compared with extracts of resting cartilage. Preliminary analyses also show a correspondingly higher acid-soluble phosphate content in extracts of the transforming zone; the significance of this is not yet clear.

With regard to physical changes between these two regions, it has been noted that the residues after homogenization and aqueous extraction, which are mainly collagen, vary in their swelling properties. The collagen of the resting zone takes up nearly 50% more water than collagen from the transforming zone.

CONCLUSIONS

At the present relatively early stage in this work the following conclusions may be drawn:

1) Despite many years of work in various laboratories on the constitution and metabolism of connective tissue, practically nothing is known of the relationship between a particularly important kind of connective tissue, ossifying cartilage, and the uptake of normal and abnormal metal cations. The field remains an almost limitless area for research.

2) The calf scapula is a useful source of large amounts of such cartilage for study of the physical and chemical transformations taking place during the early stages of bone formation.

3) The demonstration of differences in the sialic acid and organic phosphate content, as well as in the swelling

properties of the collagen-rich portions of these cartilage fractions, forces us to look beyond the mucoproteins in our investigation of the physical and chemical changes involved in calcification and ossification.

STUDIES ON LYSOSOMES

Y. E. Rahman

PURPOSE AND METHODS

During the last two decades, due to the introduction of two new techniques, namely the ultracentrifugation and the electron microscope, to the biochemical studies of cells, tremendous progress has been made in the field of cell physiology and pathology on an ultrastructural level.

The lysosome, a new subcellular particle, was discovered in the cytoplasm of rat liver cells by a group of Belgian scientists in 1955 as the consequence of the new technical applications cited above. During the subsequent years, as a result of studies done in several laboratories throughout the world, the findings on the existence of these new particles in tissues other than liver, and in animal species other than mammals, as well as the important role played by lysosomes in phenomena such as intracellular catabolic processes and phagocytosis, seem to be well established; however, the functioning and the control mechanisms as well as the true meaning of the functions attributable to lysosomes at the present time are entirely unknown.

An attempt to understand further the functional and control mechanisms of these lysosomes in terms of biochemistry, physiology, and pathology is being made in the present study, with particular attention being paid to the behavior of these lysosomes of mammalian tissues in the presence of various ionizing radiations. Therefore, my studies of lysosomes were focused mostly on the so-called radiosensitive tissues such as spleen and thymus.

Methods being used are essentially limited to the following: 1) ultracentrifugation with density gradients for isolation of subcellular particles, 2) enzyme determinations (since each group of particles has a different enzyme complement, one can use these enzymes as biochemical markers for the identification of a specific group of particles), and 3) electron microscopy for morphological identification of isolated subcellular particles.

PROGRESS REPORT

The existence of lysosomes in the thymus was demonstrated originally in this laboratory. They were

REFERENCE

1. Kuettner, K. E. and A. Lindenbaum. Heparinic acids: determination of equivalent weights and sulfate-to-carboxyl ratios. *Science* **144**, 1228-1229 (1964).

found mostly (if not exclusively) in the cytoplasm of the reticular cells of the thymus and were seldom seen in the lymphocytes of the same tissue. This was simultaneously studied by means of electron microscopy as well as by determination of lysosomal enzymes.

After whole body X-irradiation, spleen and thymus of various mammals showed a striking increase in the specific activity of some of their enzymes. (These enzymes were later found to belong to the lysosomal particles.) This was reported by several workers, and no satisfactory explanation was offered. Similar studies were completed in this laboratory, and results show that after a single dose of 200 or 1,000 R, rat spleens and thymuses show a steady increase of their lysosomal enzymes (acid phosphatase and β -glucuronidase) with time after irradiation; but these increases could only be seen if the enzyme activities were expressed on the basis of unit nitrogen, and were unchanged if activities were expressed as total activity of the whole organ. This means that X-irradiation did not affect the enzymes *per se*, but after the degeneration and subsequent disappearance of the lymphocytes (which contain relatively few lysosomes) during the postirradiation period, the lysosomal enzymes remained along with most of the reticular cells in these tissues. Thus, the increases in specific activities of these enzymes are merely the result of a selective retention of the reticular cells after irradiation.

Because of the minute number in all tissues, the isolation of lysosome particles has become one of the most challenging problems to be solved. Based on the selective retention of the reticular cells in thymuses after X-irradiation, an attempt was made to isolate the lysosomes from thymuses of irradiated rats by the sucrose density gradient centrifugation method, and a much purer lysosome fraction than the one from unirradiated thymuses was obtained.

Experiments were also done to investigate the possible action of irradiation on the lysosomes of radiosensitive tissues. It was found that X-irradiation renders the membrane of these particles more fragile; hence, their enclosed enzymes are more readily released. This increased fragility was not seen in thyroidectomized rats. Injection of rats with thyrotropic hormone or treatment

of spleen homogenate with L-thyroxine *in vitro* produced an effect similar to that of X-irradiation. Thus, the X-ray effect on the lysosomal enzyme release cannot be attributed to a direct action on membranes of these particles, but is apparently mediated through the thyroid hormone.

To investigate the effect of localized internal irradiation on lysosomes of various tissues (radioresistant as well as radiosensitive), experiments were done in collaboration with Dr. A. Lindenbaum with colloidal plutonium, $\text{Pu}^{239}(\text{IV})$, intravenously injected into a number of rats. Tissue fractionation studies were made of their livers 1 day, 8 days and 28 days after the injection; the subcellular distribution of the plutonium was found to be nearly identical with the distribution of the acid phosphatase, which was taken as an enzyme marker for lysosomes. But the fragility of lysosomes, based on studies of the release of acid phosphatase from liver and spleen lysosomes, showed no difference when compared to that of the control animals. From the results of these experiments, good evidence was added on one hand to the suggested role played by lysosomes in the process of phagocytosis of colloidal material by the liver, and on the other hand, additional indirect evidence was pro-

vided for the fact that irradiation has no direct action on lysosomal membranes.

Along with the preceding experiments, electron microscopic studies were done on livers of rats having received plutonium intravenously. Two interesting findings resulted from this study: 1) degenerated lipid bodies were seen in the cytoplasm of hepatic and Kupffer cells 1 day and 8 days after Pu^{239} injection; these bodies, relatively small after 1 day, almost filled up the entire Kupffer cells 8 days after injection; and 2) fragments of erythrocytes were seen in the cytoplasm of hepatic cells throughout sections of liver 1 day after plutonium injection. This indicates the existence of erythrophagocytosis by hepatic cells under the above experimental conditions; the mechanism and the meaning of this process are, however, unknown.

Studies have been undertaken on the release of lysosomal enzymes under different physical and chemical conditions (i.e., pH, temperature and phospholipase C) from spleen, thymus and liver of rats. Acid phosphatase has been studied so far, and acid ribonuclease is currently being studied. The pattern of release of these enzymes from the liver differs from the pattern found in the spleen and thymus.

ENZYMATIC INTERCONVERSION OF 5'-METHYLTHIOADENOSINE AND S-ADENOSYLMETHIONINE

F. Schlenk

PURPOSE AND METHODS

5'-Methylthioadenosine (Figure 2) is formed from S-adenosylmethionine when the latter acts as a donor of its propylamino side chain. In the presence of specific decarboxylating and condensing enzymes, the propylamino group is combined with putrescine to yield spermidine (Figure 3 A). By addition of another propylamino unit to spermidine, spermine is formed. Another reaction leading from S-adenosylmethionine to 5'-methylthioadenosine is the enzymatic formation of homoserine lactone and homoserine (Figure 3 B).

FIG. 2.—5'-Methylthioadenosine

The almost ubiquitous cellular occurrence of spermidine and spermine, and the frequent occurrence of homoserine in plant material suggest that 5'-methylthioadenosine should be present in cells and tissues in corresponding quantities. However, despite numerous

FIG. 3.—Enzymatic cleavage of S-adenosylmethionine to 5'-methylthioadenosine.

surveys, the compound has never been found in significant quantity except as a product of inadvertent chemical decomposition of the sulfonium precursor. The low level of the nucleoside in cells might be explained by rapid catabolism, but a search for the required enzymes showed only sporadic occurrence of such reactions. On the other hand, our observations on the regeneration of S-adenosylmethionine from S-adenosylhomocysteine after transmethylation pointed to a mechanism of cellular economy by reutilization of the core of the enzymatic donor molecule. It appeared possible that a similar regeneration of S-adenosylmethionine may take place from 5'-methylthioadenosine. This would explain the formation of relatively large quantities of spermidine, spermine, and homoserine by the action of catalytic amounts of S-adenosylmethionine.

PROGRESS REPORT

The hypothesis was tested with cells of the yeast *Candida utilis*. In such studies yeasts usually have the advantage that the level of the sulfonium compound is high, and that it is accumulated in the vacuole. When 5'-methylthioadenosine was incorporated into the culture medium, a significant increase in the intracellular level of S-adenosylmethionine was achieved. While the increase was indicative of a conversion, a nonspecific precursor effect or mere stimulation of the endogenous synthesis of the sulfonium compound could not be excluded. Only tracer experiments could verify the specific precursor activity of 5'-methylthioadenosine. For this, the nucleoside was prepared in three labeled modifications: 5'-methylthioadenosine-S³⁵, 5'-methylthioadenosine-adenine-H³, and 5'-methylthioadenosine-C¹⁴H₃.

The first two modifications gave the expected isotope recovery in the sulfonium compound, while the methyl-labeled nucleoside led to S-adenosylmethionine with less than the expected specific radioactivity. The explanation

is that the methyl group enters into transmethylation as soon as the sulfonium compound is formed; it is readily restored from unlabeled methyl precursors. After transfer into the vacuole the compound remains metabolically inert.

It is not yet known which four-carbon amino compound reacts with 5'-methylthioadenosine to yield the α -aminobutyryl moiety of the sulfonium compound. Exploratory experiments with labeled aspartic acid, labeled homoserine, and with threonine have not yet led to conclusive results. When these substances were provided in the culture medium, they were rapidly diluted by the intracellular pool of unlabeled nitrogenous compounds. Attempts to use starved cells and four-carbon amino acids as the sole source of nitrogen were complicated by the sluggish assimilation of homoserine and threonine. A further complication is provided by the facile interconversion of all four-carbon amino acids in the yeast cell. Further research is needed to elucidate details of the mechanism. Of particular interest is the apparent formation of an energy-rich sulfonium compound from low-energy precursors. Such reactions are known to require the interaction of adenosinetriphosphate. A detailed investigation with cell-free enzymes is suggested; it should bring to light new mechanisms.

The present stage of this investigation could be reached only by the use of labeled precursors. The procedures for their biosynthesis, chromatographic isolation and analysis have enriched the methodology of sulfonium compound biochemistry. Improvement of yeast culture methods has been achieved, and incidental experiments on the specificity of a variety of sulfur-containing biochemical compounds have been carried out. The details are described in a recent publication.⁽¹⁾

REFERENCE

1. Schlenk, F. and D. J. Ehninger. Observations on the metabolism of 5'-methylthioadenosine. *Arch. Biochem. Biophys.* **106**, 95-100 (1964).

THE METABOLIC RELATIONSHIP OF METHIONINE TO ITS SULFONIUM DERIVATIVES

Stanley K. Shapiro, Edward Balish, and Dimis J. Ehninger

PURPOSE AND METHODS

Methionine is one of the so-called essential amino acids, and it also serves as an indirect source of methyl groups for many biological compounds. In recent years it has been established that adenosylmethionine is the most important metabolic source of intact methyl groups in the biosynthesis of numerous biological compounds, including phospholipids, sterols, and methylated nucleic acids. The relationship of adenosylmethionine to methionine biosynthesis was first demonstrated

in this laboratory several years ago. At that time we described the enzyme, adenosylmethionine:homocysteine methyltransferase, which catalyzes the following reaction:

This enzyme also utilizes methylmethionine as a methyl donor. Within the last two years it has been established that adenosylmethionine is required in the only other

known pathway of methionine biosynthesis. This involves the *de novo* synthesis of the methyl group from serine via tetrahydrofolate and vitamin B₁₂.

This project has been concerned with the metabolism of adenosylmethionine and adenosylhomocysteine with particular emphasis on their relationship to the mechanism of methionine biosynthesis. The experiments involve the standard techniques of enzymology and biochemical genetics in addition to extensive use of C¹⁴, S³⁵, and H³ in studies with cell-free extracts and whole cells.

PROGRESS REPORT

The methods of purification and the properties of the enzyme of *Saccharomyces cerevisiae* that catalyzes Reaction 1 have been described previously.⁽¹⁾ It was reported that in order to obtain a linear rate of reaction with respect to time, it was necessary to preincubate the enzyme and homocysteine for 15 minutes before the addition of the methyl donor. Even with preincubation, it was difficult to obtain good curves for the rate of methionine formation as a function of homocysteine concentration. Thus, it seemed possible that homocysteine might serve two functions—that of a reducing agent as well as that of a methyl acceptor.

Experiments were carried out in which various reducing agents were added to standard reaction mixtures.⁽¹⁾ At levels of homocysteine under 0.2 μ mole per 0.5 ml reaction mixture, very little methionine was synthesized. However, in the presence of mercaptoethanol, cysteine, or thiodiglycol, there was a marked increase in the rate and absolute amount of methionine production. The most effective compound was mercaptoethanol, and optimum activity was obtained at a concentration of 0.006 *M*.

In order to clarify the effect of mercaptoethanol, standard assays were prepared in which various combinations of mercaptoethanol, enzyme and homocysteine were preincubated for 15 minutes, after which the remaining requisite components and methyl donor were added to make each reaction mixture complete for subsequent incubation. The results of these experiments (Figure 4) indicate that, whether added before or after a preincubation period, the presence of mercaptoethanol in the final incubation mixture resulted in a marked increase in the formation of methionine. The greatest effect on the formation of methionine occurred when mercaptoethanol was preincubated with both enzyme and homocysteine, and this is consistent with the fact that both the preincubation mixtures of homocysteine + enzyme and mercaptoethanol + enzyme were more effective than was the mixture of mercaptoethanol + homocysteine. It is probable that during preincubation, mercaptoethanol reduces the enzyme and homocysteine is bound to the enzyme in a manner which permits the

optimum rate of transmethylation to occur upon addition of the methyl donor.

With stable, purified enzyme and mercaptoethanol in the assay procedure, it was possible to conduct meaningful kinetic studies on Reaction 1 with the adenosylmethionine:homocysteine methyltransferase of yeast cells (*S. cerevisiae*). When the enzyme is saturated with homocysteine, the *K_m* for adenosylmethionine is 3.2×10^{-4} *M*. Similarly, when the enzyme is saturated with adenosylmethionine, the *K_m* for homocysteine is 8.6×10^{-4} *M*.

FIG. 4.—Effect of mercaptoethanol on the biosynthesis of methionine. Compounds to the left of the semicolon were preincubated at 37° C for 15 minutes. Compounds to the right of the semicolon were added after preincubation, and the complete reaction mixtures were incubated for 45 minutes. H, homocysteine; MER, mercaptoethanol; E, enzyme; AM, adenosylmethionine.

In order to obtain information concerning the order of addition of the two substrates, product inhibition studies were undertaken. When the rate of methionine formation is plotted as a function of the product, methionine, there is 50% inhibition of the reaction at levels as low as 0.4 μ mole of added methionine per 0.5 ml reaction mixture. The other product, adenosylhomocysteine, causes a very similar rate of inhibition of the reaction. Selecting the 25% level of inhibition caused by methionine, we undertook a series of experiments in which the levels of both substrates were varied in the presence and absence of this product. The same series of experiments was performed at the 25% level of inhibi-

tion caused by the other product, adenosylhomocysteine. With the cooperation of Dr. John Thomson of this laboratory, the results were analyzed according to the methods developed for kinetic studies of two-substrate systems. These calculations indicate that the first substrate bound to the enzyme is homocysteine and the last product released is methionine. The exact mechanism of the reaction is probably very complex since it may involve not only product inhibition but both product and substrate inhibition.

TABLE 2
GROWTH RESPONSES OF MUTANTS OF *Aerobacter aerogenes**

SUPPLEMENT (0.15 μ mole/ml)	MUTANT 62		MUTANT 68	
	18 Hr	24 Hr	18 Hr	24 Hr
Methionine.....	192	213	205	212
S-Adenosylmethionine.....	10	14	162	181
S-Methylmethionine.....	0	0	0	0
Homocysteine.....	31	117	75	75
Homocysteine+S-adenosylmethionine.....	123	156	146	161
Homocysteine+S-methylmethionine.....	120	192	169	172
S-Adenosylhomocysteine.....	62	178	4	10
S-Adenosylhomocysteine+S-adenosylmethionine.....	152	185	158	160
S-Adenosylhomocysteine+S-methylmethionine.....	171	210	133	186
S-Ribosylhomocysteine.....	88	86	87	81
S-Ribosylhomocysteine+S-adenosylmethionine.....	97	101	190	183
S-Ribosylhomocysteine+S-methylmethionine.....	196	232	192	210

* After incubation on a reciprocal shaker at 35° C, growth was measured with a Klett-Summerson Colorimeter. The results are expressed as Klett units at 660 m μ .

The metabolic pathways of adenosylmethionine and adenosylhomocysteine in whole cells were investigated by studies of the metabolism of these compounds by two radiation-induced methionine mutants of *Aerobacter aerogenes* that are blocked at various steps in the biosynthesis of methionine, adenosylmethionine, or both. Mutants 62 and 68 utilize adenosylhomocysteine and adenosylmethionine, respectively, for growth, and cell-free extracts of these organisms exhibit high adenosylmethionine:homocysteine methyltransferase activity.⁽²⁾

Table 2 summarizes some of the growth responses of these *A. Aerogenes* mutants. After incubation for 18 or 24 hours, mutant 62 showed about one-sixth to one-half maximal growth on homocysteine but little or no growth on adenosylmethionine or methylmethionine. Mutant 68 also showed no growth on methylmethionine and about one-third maximal growth on homocysteine. In contrast to mutant 62, however, mutant 68 grew very well on adenosylmethionine. The combinations of homocysteine + adenosylmethionine and homocysteine + methylmethionine permitted much better growth of mutant 62 than either of the compounds alone. Mutant 68 responded similarly to the combina-

tions although with respect to homocysteine + adenosylmethionine, it is not clear from the growth experiments whether this organism was responding only to the adenosylmethionine, on which it grows well, or whether it was responding to the combination. The fact that the combinations of homocysteine + adenosylmethionine and homocysteine + methylmethionine supported almost maximal growth for these mutants suggests that transmethylation occurs via Reaction 1 and produces methionine for growth.

Adenosylhomocysteine supported about one-third maximal growth of mutant 62 after 18 hours. However, there was a marked increase in the growth response when adenosylmethionine or methylmethionine was present in addition to adenosylhomocysteine. Even after 24 hours, growth on the combinations remained better, particularly when methylmethionine was the methyl donor. Ribosylhomocysteine supported less than one-half maximal growth of mutant 62 after incubation for 18 or 24 hours. There was little effect when adenosylmethionine was in combination with ribosylhomocysteine, but methylmethionine + ribosylhomocysteine resulted in a marked growth response.

FIG. 5.—Proposed interrelationships of various sulfonium compounds in the pathway of methionine biosynthesis. Solid lines represent reactions that have been demonstrated with cell-free extracts; dashed lines represent hypothetical reactions based on observations with labeled compounds and whole cells; solid vertical lines under mutant 68 and mutant 62 represent hypothetical genetic blocks in these mutants of *Aerobacter aerogenes*.

In contrast to the response of mutant 62, adenosylhomocysteine did not support the growth of mutant 68. Ribosylhomocysteine again supported less than one-half maximal growth. However, the combination of methylmethionine with either adenosylhomocysteine or ribosylhomocysteine resulted in a marked growth response of mutant 68. As with mutant 62, this clearly represents a response to the presence of both compounds. The response of mutant 68 to adenosylhomocysteine + adenosylmethionine and ribosylhomocysteine + adenosylmethionine may again represent a response to only the adenosylmethionine or to the combinations.

The *in vivo* studies reported above and the *in vitro* studies to be discussed below suggested the interrelationships of various compounds as shown in Figure 5.

The following enzymatic reactions in Figure 5 have been previously demonstrated with cell-free extracts of the same mutant cultures:⁽²⁻⁴⁾

It is proposed that mutant 68 uses adenosylmethionine via the enzymatic decomposition of adenosylmethionine to methylthioadenosine. The latter compound enters the methionine pathway at some point where it can serve as a source of the sulfur of homocysteine. In addition, adenosylmethionine serves as a methyl source for the methylation of the homocysteine to methionine. Evidence for this proposed pathway was obtained by growing mutants 62 and 68 on adenosylmethionine-S³⁵ with three levels of homocysteine. The

methionine in the presence or absence of homocysteine. Presumably, this organism is blocked after the point where methylthioadenosine or some related intermediate enters the pathway, and consequently mutant 62 does not grow on adenosylmethionine.

Mutant 62, however, utilizes adenosylhomocysteine for growth (Table 2). It is proposed (Figure 5) that mutant 62 uses adenosylhomocysteine via the enzymatic decomposition of adenosylhomocysteine to ribosylhomocysteine which may then be broken down to homocysteine which is methylated to methionine via Reaction 1. This pathway probably occurs in mutant 68 also, but it seems to require the presence of a methyl donor. Evidence for this pathway has been obtained with cell-free extracts of both organisms. Reaction mixtures containing adenosylhomocysteine, methylmethionine-C¹⁴H₃, 0.1 M phosphate buffer, pH 6.7, and enzyme extract were prepared. After incubation at 37° C for 2 hours, ribosylhomocysteine and radioactive methionine were identified in these reactions mixtures. Ribosylhomocysteine formation from adenosylhomocysteine was dependent only upon the presence of enzyme, but the formation of radioactive methionine was dependent upon the presence of adenosylhomocysteine, methylmethionine-C¹⁴H₃, and enzyme. These results demonstrate the enzymatic decomposition of adenosylhomocysteine and support the hypothesis that the resultant ribosylhomocysteine is broken down to homocysteine which is methylated to methionine.

TABLE 3
UTILIZATION OF S-ADENOSYLMETHIONINE-S³⁵ FOR
THE BIOSYNTHESIS OF PROTEIN METHIONINE*

SUBSTRATE (μmole/ml)		INCORPORATION OF LABEL INTO PROTEIN METHIONINE†	
S-Adenosyl- methionine‡	Homocysteine	Mutant 62	Mutant 68
0.15	0.48	2.8	32.5
0.15	0.15	4.6	55.6
0.20	0		82.3

* Methionine isolated as methionine sulfoxide.

† Specific activity of methionine sulfoxide × 100 divided by specific activity of S-adenosylmethionine.

‡ Specific activity of S-adenosylmethionine-S³⁵ in counts/minute/μmole: 0.15 μmole/ml, 99,000; 0.20 μmole/ml, 152,000.

protein methionine of these cells was isolated and the specific activity determined (Table 3). The methods for this procedure have been described by Shapiro *et al.*⁽⁵⁾ Mutant 68 showed over 80% incorporation of the sulfur of adenosylmethionine-S³⁵ into protein methionine in the absence of homocysteine. In the presence of homocysteine, the per cent of incorporation was significantly lower. It seems reasonable to assume that radioactive homocysteine-S³⁵ is derived from the adenosylmethionine-S³⁵ via methylthioadenosine-S³⁵ in some unknown manner and that the dilution of the specific activity of methionine depends on the concentration of nonradioactive homocysteine added to the culture medium. On the other hand, mutant 62 did not incorporate any significant amount of sulfur into protein

REFERENCES

1. Shapiro, S. K., D. A. Yphantis and A. Almenas. Biosynthesis of methionine in *Saccharomyces cerevisiae*. Partial purification and properties of S-adenosylmethionine:homocysteine methyltransferase. *J. Biol. Chem.* **239**, 1551-1556 (1964).
2. Shapiro, S. K. Utilization of S-adenosylmethionine by microorganisms. *J. Bacteriol.* **83**, 169-174 (1962).
3. Shapiro, S. K. and A. N. Mather. The enzymatic decomposition of S-adenosyl-L-methionine. *J. Biol. Chem.* **233**, 631-633 (1958).
4. Duerre, J. A. A hydrolytic nucleosidase acting on S-adenosylhomocysteine and on 5'-methylthioadenosine. *J. Biol. Chem.* **237**, 3737-3741 (1962).
5. Shapiro, S. K., P. Lohmar and M. Hertenstein. Utilization of S-adenosylmethionine for the biosynthesis of methionine. *Arch. Biochem. Biophys.* **100**, 74-79 (1963).

ULTRAVIOLET MICROSCOPY AS A TOOL FOR CYTOLOGICAL AND BIOCHEMICAL RESEARCH

George Svihla and F. Schlenk

PURPOSE AND METHODS

Ultraviolet microscopy offers an excellent opportunity to observe the location and concentration of certain naturally occurring chemical substances present in the living cell. There are limitations which must be recognized and accepted or circumvented. The ultraviolet energy used can be injurious to living protoplasm, but this effect can be kept at a minimum and may be turned into an advantage for the study of irradiation effects. Another important factor is that of size as related to concentration of ultraviolet-absorbing material. We cannot adjust the concentration of samples for optimum measurement, as is usually done in spectrophotometry. Concentrations present in the living organism are dictated by the state of life itself. Specimens for study, therefore, must be carefully selected.

The yeast cell is a good example of a suitable specimen. Its content of ultraviolet-absorbing materials lies within the range of observation by ultraviolet microscopy, and its lack of motility is an important factor in the production of useful images with the available ultraviolet energy.

The morphological and physiological changes occurring in yeasts under varied culture conditions, coupled with the availability of a rather unique ultraviolet microscope, providing rapid access to high-quality photographic images, have been the basis of our program.

It is the purpose of the program to exploit the combination of these opportunities for cytological research.

Morphological changes and changes in location and concentration of ultraviolet-absorbing materials in the living cell are followed by ultraviolet microscopy. The results are correlated with chemical analyses, chromatography, spectrophotometry, etc., of comparatively large samples.

PROGRESS REPORT

During this report period (1964) we have been concerned with some of the details of sporulation in *Saccharomyces cerevisiae*. Most cytological investigations of sporulation in *S. cerevisiae* in the past have been centered on the behavior of the nucleus, but extensive changes, apparently involving other structures such as the vacuole, mitochondria, and cytoplasmic granules, also occur. Exceptional metabolic activity is a prerequisite of such changes. Are preexisting structural and

molecular units of the vegetative cell used directly with varying degrees of modification, or are spore constituents synthesized *de novo*?

Favorable material for a study of the part played by the vacuoles was obtained by culturing cells under conditions which lead to a high concentration of the strongly ultraviolet-absorbing compound, S-adenosylmethionine, in the vacuole. Normal cells and cells with the ultraviolet-absorbing vacuoles were induced to sporulate. Ultraviolet photomicrographs (Figure 6) of samples obtained during the sporulation period showed that the vacuole is abolished at the onset of sporulation, and its contents may be observed temporarily within the old cell wall, surrounding the spores. As sporulation progresses, the material is discharged into the culture medium. It is not utilized. This is quite different from what occurs during budding, where the vacuolar material appears in the vacuole of the bud in a concentration indicating that parent and daughter share equally. Analysis of the culture medium showed that sporulation of both types of cells leads to a release of nucleic acid fragments into the medium. Thus, despite severe nutritional restrictions imposed by the composition of the sporulation medium, the cell utilizes very little, if any, of its nucleotides in assembling the ribonucleic acids of the spores.⁽¹⁾

While our chief concern in this study was with the vacuole, we observed that the cytoplasmic granules were apparently playing an important part. Consequently cells were cultured under conditions leading to cells containing large, easily observed, cytoplasmic granules. The cells were then transferred to sporulation medium where they were found to sporulate satisfactorily.

Ultraviolet photomicrographs of these cells revealed that most of the granules disappeared in the process of sporulation and that an ultraviolet-absorbing substance lay between the spores, wetting them. The quantity of this material was much greater than that seen occasionally in the sporulation of normal cells. Crushed cells, in which membranes surrounding the granules were apparently broken, showed coalesced masses of material similar to that observed between the spores. Tests with Sudan IV indicate that at least some of the granules are composed of, or contain, lipid.

At present the interpretation of the observed results is that lipid material present in the granules takes part in

FIG. 6.—Photomicrographs of *Saccharomyces cerevisiae* obtained using ultraviolet energy of 265 m μ wavelength to illustrate the effects of varied culture conditions on cell structures and the consequent effects on spore formation: a, normal cells; b, cells containing S-adenosylmethionine in their vacuoles; c, cells containing large cytoplasmic granules; d, characteristic appearance of spores obtained from normal cells and from S-adenosylmethionine containing cells after leakage of the S-adenosylmethionine from the ascus; e, the budding cell illustrates sharing of S-adenosylmethionine by parent and daughter and free floating spores surrounded by S-adenosylmethionine present in the parent; f, ultraviolet-absorbing (black) material lies between spores derived from cells with large cytoplasmic granules.

the formation of the spore coat, especially the outer layer. To clarify this, electron micrographs are being obtained. Chemical analysis of the material has not yet been undertaken. Removal of the yeast cell wall by enzyme action, as was done in our spheroplast investigation, will be used to aid in obtaining adequate quantities of relatively clean material.

Of all ultraviolet-absorbing compounds, deoxyribonucleic acid (DNA) probably holds the highest interest of biologists. Unfortunately, it has not been possible to get information on the disposition of the DNA fraction

during sporulation. One may surmise that most of it is transferred into the spores. Exploratory experiments with tritiated thymidine have not led to conclusive results in our hands, because the assimilation of this material in the presporulation medium was negligible, and the quantity of DNA in yeast cells is so small that adequate analyses are difficult.

REFERENCE

1. Svihla, G., J. L. Dainko, and F. Schlenk. Ultraviolet microscopy of the vacuole of *Saccharomyces cerevisiae* during sporulation. *J. Bacteriol.* 88, 449-456 (1964).

THE METABOLISM OF MITOCHONDRIAL PROTEINS

Robert W. Swick

PURPOSE AND METHODS

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

The array of enzymes in this particle has usually been regarded as quite constant; e.g., cytochrome oxidase activity is frequently used as an index of the mitochondrial population. Recently, however, modification of the activity of several mitochondrial enzymes by hormonal or dietary manipulation of the animal has been demonstrated. If the above hypothesis of mitochondrial formation is valid, then the rate of change of enzyme activity should be similar to the rate of synthesis of new mitochondria as measured by the incorporation of isotope. This would give an independent measure of the turnover rate of this structure.

Alanine aminotransferase was chosen for investigation for the following reasons. Its activity, when determined in cell homogenates, is subject to marked alteration. Also, there was evidence for the presence of a mitochondrial isozyme although, when this study was initiated, its existence was somewhat equivocal. Therefore, one phase of this work was concerned with the establishment of the existence of the mitochondrial form of alanine aminotransferase and with attempts to enrich and characterize it. The comparison of the changes in activity, wrought by dietary and hormonal treatment, of the individual isozymes adds a new dimension to the study of the control of metabolic processes. The rate of change in mitochondrial alanine aminotransferase activity and its relation to that of other mitochondrial enzymes whose activity can be modified and to protein turnover as measured by isotope incorporation remains to be determined.

Young adult female rats were fed diets of varying protein composition, injected with corticosteroids, made diabetic with alloxan, or irradiated with 700 R of X-rays. The animals were sacrificed 4–22 days later and hepatic alanine aminotransferase activity determined.

When it became apparent that the mitochondrial isozyme was quite unstable, it was necessary to modify the usual methods of preparation of cellular fractions and of exposure of mitochondrial enzymes for assay purposes. A 10% homogenate in 0.25 *M* sucrose-0.01 *M* K_2HPO_4 was used for enrichment of the particulate fractions by differential centrifugation. Enriched fractions were also obtained by centrifugation in a sucrose gradient. The pellets were suspended in 0.05 *M* potassium phosphate (pH 7.4)-0.025 *M* L-alanine-0.002 *M* L-cysteine and assayed 30–60 minutes later. The more stable isozyme of alanine aminotransferase found in the supernatant fraction could be assayed whenever convenient.

The low activity of the mitochondrial isozyme relative to that of other enzymes catalyzing the oxidation of NADH made it compulsory to use an uncoupled assay. The amount of pyruvate formed from L-alanine in the presence of α -ketoglutarate during a timed incubation, which was stopped by the addition of trichloroacetic acid, was estimated spectrophotometrically by the addition of lactic dehydrogenase and NADH.

PROGRESS REPORT

Enzyme assays carried out on the five fractions obtained by differential centrifugation showed a small amount of alanine aminotransferase activity in the nuclear, microsomal, and poorly sedimentable fractions. Activity in the mitochondria was higher (8% of the total) than in the other particulates, but most of the activity was in the supernatant fraction (90%). Gradient centrifugation, because it affords a cleaner fractionation, was used to show more clearly the presence of the enzyme in the mitochondria. Succinate dehydrogenase, a mitochondrial enzyme, was used to indicate the precise location of the peak of mitochondrial activity. A large peak of alanine aminotransferase activity corresponding to the nonparticulate phase and a small peak coinciding with that of succinate dehydrogenase activity were observed.

Further evidence for the existence of two alanine aminotransferases was obtained from a comparison of the chemical characteristics of the isozymes. The soluble enzyme was prepared according to a published procedure; the kinetic constants and optimum pH range observed were similar to those previously reported. We found that the rate of the reverse reaction (pyruvate + glutamate $\rightarrow$ alanine + α -ketoglutarate) was slower than the forward reaction, in contrast to earlier work. En-

richment and characterization of the mitochondrial enzyme from rat liver have proved difficult because of the extreme lability of the enzyme. Solubilization without serious loss in activity could not be effected by any of the usual methods. However, when the mitochondria were first suspended in cold, 50% glycerol-0.001 *M* L-alanine, sedimented, and resuspended in 0.05 *M* phosphate buffer-0.25 *M* L-alanine, half of the activity could be solubilized although there was little enhancement of the specific activity. Suspension first in 30% dimethyl sulfoxide was as effective as 50% glycerol.

TABLE 4
SUMMARY OF CHEMICAL CHARACTERISTICS OF
ALANINE AMINOTRANSFERASE ISOZYMES

	ENZYME SOURCE	
	Mito- chondria	Supernatant Fraction
pH optimum.....	8.0	7.2
Forward:reverse reaction.....	0.81	1.33
K_m for alanine in mM.....	5.0	34.0
K_m for α -ketoglutarate in mM.....	0.68	0.81
Stability.....	—	++

Further enrichment has not yet been achieved. Heat, pH changes away from neutrality, fractionation with ammonium sulfate or by electrophoresis or dialysis all led to a rapid loss in activity. This loss was not the consequence of dissociation of the coenzyme from the protein, since the addition of, or preincubation with, pyridoxal phosphate failed to restore activity. The enzyme could be stabilized by storage in high concentrations of glycerol, erythritol, or dimethyl sulfoxide; hence, esti-

mates of the kinetic parameters have been made using such relatively crude preparations. A comparison of the properties of the isozymes is shown in Table 4.

A recent experiment has shown that the hetero-enzyme prepared from rabbit liver mitochondria is considerably more stable. Future attempts at purification may be made using rabbit liver; other sources will also be sought.

A study of the effect of various dietary and hormonal treatments on alanine aminotransferase activity in the supernatant and mitochondrial fractions of rat liver was carried out. The isozymes reacted similarly to treatment with corticosteroids and alloxan, but failed to do so in animals either fasted or kept on a diet containing only 2% casein. Also, the mitochondrial enzyme activity returned to normal levels when an 80% casein diet was fed for three weeks and appeared to return to control levels more rapidly than did the soluble enzyme when the diet contained a mixture of essential amino acids as a protein source. In all conditions that caused a breakdown of protein, whether of endogenous or exogenous origin, the activity of the soluble isozyme was consistently elevated. Conversely, in conditions that caused a reduced catabolism of protein, alanine aminotransferase activity in the supernatant fraction was lowered. The mitochondrial isozyme did not always respond in this manner, suggesting that the isozymes are different proteins and only partly under the same metabolic controls.

It was found that the rise in enzyme activity following X-irradiation was confined to the soluble enzyme; the level of the mitochondrial form remained essentially unchanged. A similar pattern was observed in pair-fed rats; hence, the effects of irradiation on this enzyme are probably those associated with anorexia.

PHARMACOLOGY OF DEUTERIUM. I. DEUTERIUM ISOTOPE EFFECTS ON LACTATE DEHYDROGENASE

John F. Thomson and Sharron L. Nance

PURPOSE AND METHODS

To a considerable extent the toxicological basis of deuterium is the so-called primary isotope effect: bonds to deuterium will generally be broken at a slower rate than corresponding bonds to hydrogen. Thus the effect on an organism of replacement of hydrogen by deuterium would be a metabolic imbalance resulting from differential enzyme inhibition. It is obvious of course that the toxic effects of deuterium cannot be related to its inhibitory action on any one enzyme; nevertheless it is instructive to study isotope effects on purified

enzyme systems *in vitro* for two reasons: a) to ascertain whether the inhibition of a specific enzyme might be important in the organism, and b) to obtain information on the mechanism of action of an enzyme by studying the influence of deuterium on its kinetics.

Recent emphasis has been on the effects of replacement of either the transferable or nontransferable hydrogen in the 4-position of the pyridine ring of reduced nicotinamide-adenine dinucleotide (DPNH) on the kinetics of lactate dehydrogenase (LDH). Some of the information obtained with skeletal muscle LDH has

been reported previously.⁽¹⁾ This communication is concerned with additional studies on skeletal muscle LDH, and preliminary studies on heart LDH.

Details of the procedures used have been described elsewhere.⁽¹⁾ Briefly, 25 different combinations of coenzyme (DPNH, A-DPND, or B-DPND) and substrate (pyruvate) are mixed with the enzyme. The reaction is followed for about 3 minutes by observing the disappearance of the coenzyme, indicated by decreased ultraviolet absorption at 340 mμ. The initial velocity of the reaction, v , is calculated, and for each of 5 different substrate concentrations (S), $1/v$ is plotted against the reciprocal of the coenzyme concentration

PROGRESS REPORT

Skeletal Muscle LDH. Effect of Temperature on Isotope Effects

The earlier work with this enzyme was carried out at 25° C. We have repeated a number of these experiments at other temperatures ranging from 13 to 37° C. At 37°, there was evidence of denaturation of the enzyme, and the data obtained at this temperature will not be further discussed.

The results in the range of 13 to 34° are summarized in Table 5. The ϕ coefficients, the rate constant k_2 , and the equilibrium constant $K_1 (= k_2/k_1)$ are shown in this table as the ratios of values obtained with deuterated

TABLE 5
INFLUENCE OF TEMPERATURE ON DEUTERIUM ISOTOPE EFFECTS IN SKELETAL MUSCLE LACTATE DEHYDROGENASE*

Temperature (° C)	ϕ_0	ϕ_1	ϕ_2	ϕ_{12}	K_1	$1/k_2$
A-DPND						
13.....	1.79±0.03	0.72±0.03	1.32±0.04	2.52±0.13	1.91±0.09	0.38±0.03
19.....	1.90±0.17	0.93±0.22	1.37±0.09	2.04±0.31	1.51±0.21	0.61±0.17
25.....	1.46±0.17	0.90±0.09	1.56±0.10	1.55±0.78	0.98±0.13	0.91±0.08
31.....	1.60±0.22	0.85±0.39	1.88±0.12	1.62±0.27	0.86±0.14	1.00±0.62
34.....	1.55±0.09	0.61±0.33	1.53±0.62	1.78±0.11	1.16±0.07	0.80±0.37
B-DPND						
13.....	1.26±0.07	0.76±0.06	0.81±0.06	1.07±0.15	1.32±0.17	0.58±0.07
19.....	1.15±0.12	1.53±0.25	1.08±0.08	0.98±0.17	0.91±0.38	1.67±0.14
25.....	0.81±0.08	1.20±0.49	0.90±0.12	0.56±0.21	0.64±0.26	1.91±0.85
31.....	0.97±0.11	1.20±0.27	1.28±0.07	0.83±0.17	0.64±0.12	1.86±0.52
34.....	0.65±0.07	1.96±1.10	1.35±0.05	0.82±0.15	0.61±0.10	3.21±1.29

* Data are ratios of constants measured with the deuterated coenzyme to those measured with DPNH, with standard errors.

(C). The slope and intercepts of the 5 lines are plotted against $1/S$, and from the slopes and intercepts of the secondary plots, the ϕ coefficients of the general rate equation are obtained:

$$\frac{E_0}{v} = \phi_0 + \frac{\phi_1}{C} + \frac{\phi_2}{S} + \frac{\phi_{12}}{SC}. \quad (1)$$

E_0 is the enzyme concentration, and the ϕ 's are various combinations of rate constants.

For both of the LDH heteroenzymes, only two of the individual rate constants, k_1 and k_2 , can be estimated directly; these are the forward and reverse rates of binding of the coenzyme to the enzyme:

$$E + C \xrightleftharpoons[k_2]{k_1} EC. \quad (2)$$

The forward rate, k_1 , is given by $1/\phi_1$, and k_2 is equal to $\phi_{12}/\phi_1 \phi_2$.

coenzyme to those for DPNH. Values greater than 1 indicate a normal isotope effect, i.e., a slower rate with deuterium than with hydrogen.

Although these ratios are in many cases associated with rather high standard errors, they still permit a reasonable delineation of the variation in isotope effects with temperature under four limiting experimental conditions: a) when the system is saturated with respect to both coenzyme and substrate, the isotope effect is given by the ϕ_0 ratio; b) when the system is saturated with substrate, the isotope effect for very low concentrations of coenzyme is given by the ϕ_1 ratio; c) at the opposite extreme, saturation with coenzyme and low substrate, the ϕ_2 ratio gives the isotope effect; and d) when both substrate and coenzyme are present at low concentrations relative to ϕ_2 and ϕ_1 constants, the isotope effect is given by the ϕ_{12} ratio.

Thus for A-DPND, the deuterated coenzyme in

which the hydrogen is transferred to pyruvate in the course of the reaction, the isotope effect at saturating conditions (ϕ_0 ratios) is normal, but decreases as the temperature is increased. Similarly, when the substrate concentration is low, the effect is normal regardless of whether the coenzyme concentration is high (ϕ_2 ratios) or low (ϕ_{12} ratios), although the effect is increased with increasing temperature in the former case and decreased in the latter. The ϕ_1 ratios, however, show that at pyruvate saturation the direction of the isotope effect is changed at low coenzyme concentrations at all temperatures.

TABLE 6
APPARENT ENERGIES OF ACTIVATION FOR THREE
KINETIC CONSTANTS OF SKELETAL MUSCLE
LACTATE DEHYDROGENASE*

	DPNH	A-DPND	B-DPND
$1/\phi_0$ (V_{max})	9100 ± 310	11900 ± 1060	12200 ± 2510
$1/\phi_1$ (k_1)	7800 ± 2050	5900 ± 1520	3100 ± 1600
k_2	16500 ± 2560	7500 ± 2220	6100 ± 1760

* Data are given in cal/mole with standard errors.

The situation is not so clear for B-DPND. The ϕ_0 ratios show a decrease from a normal effect at low temperatures to an inverse effect at high temperatures, whereas the ϕ_1 ratios behave oppositely. That is, at high pyruvate concentrations, lowering the coenzyme concentration changes the direction of the isotope effect from normal to inverse at low temperatures, but from inverse to normal at high temperatures. When the pyruvate concentration is low, however, decreasing the coenzyme concentration from high (ϕ_2) to low (ϕ_{12}) changes the isotope effect from inverse to normal at low temperatures, and from normal to inverse at high temperatures.

For both A-DPND and B-DPND it seems to be generally true that increasing the temperature decreases the ratio when the concentrations of both of the reactants are either very high or very low, but increases the ratio when the concentration of one or the other is very low.

As pointed out earlier,⁽¹⁾ the k_1 ($1/\phi_1$) and k_2 ratios at 25° indicated that A-DPND was bound to and released from the enzyme more rapidly, and B-DPND more slowly than DPNH. It can be seen that the temperature effects are generally similar on both rates and for both deuterated homologues, except that at 13° C B-DPND is bound and released more rapidly than DPNH. The ratios of the equilibrium constant for this step, K_1 , decrease in both cases with increasing temperature.

Apparent activation energies were calculated for k_1 , k_2 , and ϕ_0 (equal to the reciprocal of the maximum velocity). The data are shown in Table 6. The standard errors of these determinations are large, but it seems clear that replacement of hydrogen by deuterium results in a decreased activation energy for coenzyme binding (k_1) and release (k_2), but an increased activation for hydrogen transfer (ϕ_0).

Heart Muscle LDH

The experiments with the heart heteroenzyme have been restricted thus far to studies at 25°. As indicated above, A-DPND was bound to the muscle enzyme more rapidly than was DPNH, in contrast to the behavior observed with other dehydrogenases (e.g., malate and alcohol), and it was of interest to find out whether the two LDH enzymes resembled one another in this respect.

The only important difference between the two, at least at 25°, was in the ϕ_2 ratios. For both A-DPND and B-DPND the ϕ_2 ratios were normal with heart LDH, whereas they were inverse with skeletal muscle LDH. Thus at low concentrations of pyruvate and high concentrations of coenzyme, the reaction catalyzed by heart LDH would proceed more slowly with the deuterated coenzymes, whereas the reaction catalyzed by skeletal muscle LDH would proceed more rapidly.

REFERENCE

1. Thomson, J. F., J. J. Darling and L. F. Bordner. Isotope and solvent effects of deuterium on rabbit-muscle lactate dehydrogenase. *Biochim. Biophys. Acta* **85**, 177-185 (1964).

PHARMACOLOGY OF DEUTERIUM. II. EFFECTS OF DEUTERIUM OXIDE ON ACTIVE TRANSPORT OF GLUCOSE

David A. Bray,* Sharron L. Nance, and John F. Thomson

PURPOSE AND METHODS

When heavy water is fed to mammals, marked physiological changes take place. The toxicity of D_2O at about 30% replacement of body water is shown by weight loss, anemia (in rats), leucopenia (in mice), impaired kidney function, hypertrophy of the liver, central nervous system disturbances, and altered carbohydrate metabolism characterized by hypoglycemia and decreased glucose tolerance.⁽¹⁾

The clearance by the kidney of *p*-aminohippuric acid (PAH) is altered, although the capacity of kidney slices to concentrate PAH *in vitro* is unaffected by concentrations of D_2O that would be lethal to an animal. However the uptake of PAH is markedly inhibited by 100% D_2O .

Another system in which accumulation of a solute takes place against a concentration gradient is the transport of glucose from the lumen of the small intestine. We have carried out experiments to study the effect of D_2O on this active transport system.

A modification of the Wilson-Wiseman technique⁽²⁾ was employed in these studies. Female hamsters were anesthetized with ether and their abdomens were opened. The small intestine was cut at the stomach and the cecum and perfused with cold Ringer solution. The perfused intestine was removed from the animal, and three segments of jejunum, about 3 cm long, were cut off and immersed in ice-cold Ringer solution. The segments were everted, filled with Ringer solution containing 0.5% glucose, tied off, and placed in a Warburg flask containing 5 ml of the same medium. The flasks were gassed with oxygen, then shaken in a 37° C water bath for varying periods of time, during which the oxygen consumption was measured. The intestinal sacs were then removed, and the concentration of glucose

in the sac and in the suspending medium was measured.

In some experiments $Na^{22}Cl$ was placed either inside the sac or in the suspending medium in order to measure the sodium fluxes. In other experiments arabinose was added to the incubation medium in order to study the accumulation of a sugar that is not actively transported.

PROGRESS REPORT

The experimental work as yet is incomplete. Nevertheless some conclusions can be safely drawn from the data now available:

1. In 100% D_2O , the active transport of glucose, measured as the ratio of serosal to mucosal (i.e., inside to outside) concentrations, is inhibited 50 to 60%, depending on the time of incubation.

2. The uptake of arabinose is inhibited only about 10% in D_2O .

3. The oxygen consumption of the intestinal segment is virtually unaffected by D_2O .

4. The outward (serosal to mucosal) sodium flux is unaltered in D_2O .

5. The inward (mucosal to serosal) sodium flux is decreased about 10%. Since a large fraction of this movement is the result of passive diffusion (which as pointed out above would not seem to be affected), it appears that D_2O has an appreciable effect on the "sodium pump." Because of uncertainties in some of the measurements, particularly volume changes inside the sac, the magnitude of this effect is not known.

REFERENCES

1. Thomson, J. F. Biological Effects of Deuterium. Pergamon Press, New York, 1963.
2. Wiseman, G. Sac of everted intestine technic for study of intestinal absorption *in vitro*. Methods in Medical Research, Vol. 9. Year Book Medical Publishers, Inc., Chicago, 1961, pp. 287-292.

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THE SYNTHESIS OF PROPIONIC ACID BY THE PROPIONIBACTERIA

Edward J. Wawzkiewicz and Robert W. Swick

PURPOSE AND METHODS

Propionic acid plays an important role in a number of biological processes. It is, for example, produced by bacteria in the digestive tract of ruminants, who can oxidize it as an energy source or incorporate it into higher, odd-numbered fatty acids. Conversely, pro-

pionic acid can arise in nature by the beta oxidation of higher, odd-numbered fatty acids. It can also arise by the degradation of branched chain aliphatic amino acids and by the reduction of certain intermediates of carbohydrate metabolism. Although the pathway of its formation by the propionibacteria has been extensively

investigated, some aspects of that process remain unclear. The penultimate step in the production of propionic acid by these microorganisms is the transfer of a carboxyl group from methylmalonyl-coenzyme A to pyruvate with the concomitant formation of oxalacetate and of propionyl-coenzyme A. Methylmalonyl-coenzyme A arises from succinyl-coenzyme A by an isomerization reaction, the detailed mechanism of which still remains obscure. Free propionate is not involved in the reaction, nor is acrylate, another substance once thought likely to take part. However, under certain circumstances acrylate-C¹⁴ can be converted to succinate-C¹⁴. Partially purified, active cell extracts bring about this conversion if provided with adenosine triphosphate, coenzyme A, reduced nicotinamide adenine dinucleotide and oxalacetate. It thus appears that acrylic acid is esterified to acrylyl-coenzyme A, reduced to propionyl-coenzyme A and then converted to succinate by transcarboxylation and isomerization. This sequence had not been demonstrated in these organisms previously and is of current interest because it implies a pathway of propionate synthesis in the propionibacteria alternate to that involving methylmalonyl-coenzyme A.

A spectrophotometric assay of propionyl-coenzyme A dehydrogenase, the enzyme which catalyzes the reduction of acrylyl-coenzyme A to propionyl-coenzyme A, has been developed to follow the enrichment of the

enzyme in fractionated extracts of the propionibacteria. We have proposed to attempt to purify this enzyme and to ascertain the role of acrylyl-coenzyme A in the metabolism of these microorganisms.

PROGRESS REPORT

We have subjected cell-free extracts of *Propionibacterium shermanii* to various protein fractionation techniques. Precipitation at concentrations of ammonium sulfate between 60 and 75% saturation at 0° brings down protein of specific activity 2 to 3 times higher than that of the protein present in the crude extracts. Fractionation of this ammonium sulfate precipitate either by passage through Sephadex G 200 columns or by adsorption and elution from DEAE-cellulose has on occasion yielded material of specific activity 60 times that of crude extract protein. Combination of both fractionation procedures has unfortunately not as yet led to the expected further enhancement of activity. Results of a preliminary experiment suggest that propionyl coenzyme A dehydrogenase may be an inducible enzyme. At least its specific activity in extracts appears to vary with the age of the cells from which the extracts are prepared and hence with the composition of the cells' external and internal milieu. We are studying this phenomenon and also surveying the entire group of propionibacteria for the presence of the enzyme.

AN ELECTRONICALLY-CONTROLLED CONTINUOUS CULTURE DEVICE

William J. Eisler and Robert B. Webb

PURPOSE AND METHODS

Several devices for growing microbial populations under steady state conditions have been described.⁽¹⁾ All of these devices were deficient in several respects for either routine continuous culture experiments or for some specialized experimental applications. We have designed and constructed a photocell-controlled instrument that meets the following requirements: 1) Utilization of ordinary commercially available chemostat glassware. 2) Provision for adequate aeration through bubbling of the culture. 3) Maintenance of population size to within $\pm 3\%$. 4) Maintenance of population densities from less than 10^7 cells/ml to 5×10^8 cells/ml. 5) Continuous record of growth rates over small increments of time. 6) Simple, sterilizable nutrient control mechanism.

PROGRESS REPORT

In this device, as in those previously described, the turbidity of a continuous culture is maintained at some preselected value by controlling the rate of influx of fresh nutrient solution into the culture while the total culture volume remains constant. The culture volume is held constant by means of an overflow siphon from the growth tube.

The growth tube is the Novick design, and the nutrient feed system is the Kubitschek modification of the Novick instrument, utilizing a modified Mariotte bottle and a single capillary for maintaining a constant nutrient flow rate.⁽²⁾ We have modified the feed system by increasing the size of the capillary to yield flow rates greater than those required to maintain the culture in a steady state. Nutrient flow is then restricted to requirements by a solenoid. A composite view of this device is shown in Figure 7.

Figure 8 is a block diagram of the complete system. The bridge contains two cadmium sulphide photocells. CDS1 is the detector cell and is employed as a nephelometric device. CDS2 monitors the light source and

compensates for changes in the intensity of the exciter lamp. Bridge balance is accomplished by adjusting a mechanical slit and potentiometer R1 for a given culture turbidity.

The electronics are cycled by timer T1 which is a 1-rpm synchronous motor and is mechanically linked to SW1 and SW2. Prior to SW1 being activated the following conditions exist.

1. The aeration bubbler is on.
2. The shutter is closed.
3. The nutrient flow solenoid valve is closed.
4. The amplifier and meter relay are gated off.

The contactors driving SW1 can be set for two monitoring cycles during the one-minute period of T1. If desired, one contactor may be removed and the second set for a single monitor event for a one-minute cycle.

1. T1 momentarily energizes SW1.
2. The aeration bubbler is turned off.
3. The shutter opens.
4. The amplifier and meter relay are gated on following a 10-second delay. This assures that all bubbling action has been completed.

The following events occur if a contact closure of the meter relay M1 is made.

1. If a turbidity reading is above the preset level, the meter relay contact is activated.
2. The automatic reset timer T2 is turned on. T2 has a maximum time range of 60 seconds; however, the range is limited by the cycle duration. Preset time may be accurately set to one-second increments.
3. The nutrient flow solenoid valve opens and feeding is started for the duration of preset time.
4. The amplifier and meter relay are gated off.
5. The aeration bubbler is turned on.
6. The shutter is closed.
7. The event recorder and digital counter record one event.

The amplifier continues to sample, anticipating either a contact closure from the meter relay or SW2 being opened by T1. SW2 can be programed for either one or two cycles in one minute. In either case SW2 trips open

FIG. 7.—An electronically-controlled continuous culture device

at a preset interval (15, 20, or 25 seconds) after SW1 is energized, thereby ending the monitoring portion of the cycle.

1. The meter relay contacts are not activated if a turbidity reading is below the preset level.
2. T1 opens SW2.
3. SW2 resets the electronics for the beginning of a new cycle.

Figure 9 is a view of the detector assembly with the growth tube (A) and overflow tube (B) in position with the clamp (C) open. The clamp is internally cushioned with rubber and when closed holds the growth tube securely. The detector cell (D) is exposed in this open position.

The detector system shown, in Figure 10, consists of a second photocell (A). This is the monitor cell and it views the exciter lamp. The exposure level is set by a mechanical slit (B). The slit is adjustable and has a calibrated scale allowing settings to be accurately reproduced.

The exciter lamp (C) operates continuously and is energized by a powerstat and step-down transformer. Instrument sensitivity can be adjusted by varying the

FIG. 8.—A block diagram of the complete system

lamp intensity. The entrance mirror (D) collimates the light beam to the growth tube. It is adjustable and is positioned for maximum light intensity. An in-line exit port (E) allows the collimated beam to disperse outside the range of the detector. This prevents the light beam from reflecting and causing a false reading.

A solenoid operated shutter (F) is actuated to interrupt the light beam. When cells exhibit a photosensitive reaction, irradiation can be held to a minimum by controlling shutter operation to conform with the monitoring cycle. Operation is optional through a toggle switch.

FIG. 9.—Growth tube, overflow tube, and detector assembly

Cells can be further shielded from undesired wavelengths by placing an absorbing solution in cell compartment (G).

Heat generated by components in the detector system is expelled through the louvered cover by fan (H). As an additional safeguard the clamp assembly is insulated from the lamp compartment with micarta spacers. This

prevents a temperature rise in the growth tube by conduction through the metal enclosure.

CONCLUSIONS

Experimental data obtained to date have shown this instrument to surpass initial performance expectations.

FIG. 10.—A detailed view of the detector system

Minor mechanical modifications to the detector system should make possible significant applications in scientific and medical fields. Considering its unique aspects of design, unavailability through commercial channels and high performance standards, we as inventors have applied for patent approval.

We are indebted to Dr. H. E. Kubitschek for his interest in the design of this instrument and for his helpful suggestions.

REFERENCES

1. Novick, A. Growth of bacteria. *Ann. Rev. Microbiol.* **9**, 97-110 (1955).
2. Kubitschek, H. E. Modifications of the chemostat. *J. Bacteriol.* **67**, 254-255 (1954).

NITRIC OXIDE AND RADIATION SENSITIVITY

E. L. Powers

PURPOSE AND METHODS

The question of the action of nitric oxide in modifying radiation sensitivity has never been fully resolved. In wet metabolizing systems, as bacteria,⁽¹⁾ ascites tumor cells,⁽²⁾ and plant seeds,⁽³⁾ nitric oxide clearly exerts a potentiating action on radiation sensitivity. The increases in sensitivity are about the same in magnitude as those caused by oxygen, and for this reason equivalence in action of these two agents has been postulated. On the other hand, in dry plant seeds⁽³⁾ and in dry bacterial spores⁽⁴⁾ nitric oxide clearly has a protective effect, with radiation sensitivity being reduced one-half in the spores. This protective effect has been

The experiments were set up in the manner reported by Tallentire and Powers.

PROGRESS REPORT

Figure 11 shows the effect of water content on the action of nitric oxide. The ordinate is the value of the inactivation constant, our measure of radiation sensitivity, and the abscissa is vapor pressure of water in millimeters of mercury to which the cells are equilibrated. The nitric oxide effect is measured in two experimental conditions—nitric oxide present during the irradiation and exposure of the cells irradiated in N₂ to NO after irradiation and prior to exposure to oxygen.

FIG. 11.—Radiation sensitivity (as inactivation constant) of bacterial spores as determined by water content when irradiated in the presence of 10% NO in N₂ (open circles) and when irradiated in N₂ followed by treatment with NO (closed circles). The solid lines represent the results of Tallentire and Powers for the three standard radiation conditions: top line, irradiation in the presence of O₂; middle line, irradiation in N₂ followed by exposure to O₂; bottom line, irradiation in N₂ followed by saturation in H₂O prior to exposure to O₂.

associated definitely with the removal by nitric oxide of radiation-induced free radicals, which, if they were present in the cell to combine with oxygen, would cause biological damage. In wet bacteria the concentration of NO has been shown to be important, with high concentrations (>20%) being protective.⁽⁵⁾ These different kinds of results have not been reconciled, although Sparman did show that nitric oxide changes from a protective compound to a sensitizing one in *Agrostis* seeds as the water content of the seeds increases.

The studies in bacterial spores were done with cells in equilibrium with about 1 torr water vapor. It was obviously necessary to measure the action of nitric oxide in spores containing varying amounts of water.

The experiments that have been reported previously are in accord with these results. The observations of Powers *et al.*⁽⁴⁾ were made on spores that were in equilibrium with about 1 torr water. On the graph at 1 mm Hg note that when nitric oxide is present after irradiation a level of sensitivity is seen that is below that obtained when nitric oxide is present during irradiation, which, in turn, is below that seen when only nitrogen is present during irradiation and oxygen treatment follows. These were the results previously reported, namely, that nitric oxide may have two actions. It is protective when it acts after irradiation in the absence of oxygen, but is sensitizing when present during irradiation. When the gas is present at the time of irradiation (when both these

actions take place) the net effect is a degree of sensitivity intermediate between full protection and the N_2 -level of sensitivity) because the sensitizing action is less than the protective one.

We note that as the water content of the spores decreases, the level of protection induced by NO after irradiation decreases slightly and then becomes invariant with respect to water content (closed circles, Figure 11). This level is coincident with that seen when the spores are treated with water after irradiation and prior to oxygen exposure (lowest solid line).

As the cells become dryer, the sensitizing action of nitric oxide present during the irradiation interval increases so that at an equilibrium vapor pressure of about 10^{-5} torr, the cells are approximately three times as sensitive in the presence of nitric oxide (open circles) as they are when nitric oxide is given postirradiation. The potentiating effect of NO on radiation sensitivity becomes very large as the cells become very dry.

As equilibrium vapor pressures increase from 1 mm Hg, we note that the action of nitric oxide during irradiation steadily decreases, and at 22 mm (saturation) the net effect of nitric oxide presence during irradiation might be a protective rather than a sensitizing one.

COMMENT

These results help us understand the action of nitric oxide in the wetter systems. Recall that as seeds are made wet NO potentiates the radiation effect. In metabolizing ascites cells, it is potentiating; in metabolizing bacterial cells, it is potentiating if the concentration is low; but in the spore, the potentiating effect steadily decreases as the cells become wetter, and in the very wet spore it disappears entirely. The difference between the spores on the one hand and the grass seed, ascites cell and vegetative bacteria on the other is that the spore does not metabolize, whatever its water content, whereas metabolism, or, certainly, metabolic readiness, exists in all of the others. In the inert spore, there is substantial reason for believing that the effects observed are on the physicochemical, rather than the biochemical or physiological, level. We interpret these results, then, as indicating that the potentiating effect of nitric oxide observed in the other systems is not physicochemical in nature, but, rather, is associated with biochemical activities in these cells. It is interesting to note that in the seed the net effect of nitric oxide presence changes from a protective to a sensitizing one at that level of water content at which respiration begins. And in

yeast⁽⁶⁾ the respiratory deficient "petite" shows the anoxic response in the presence of NO, while the sufficient form is sensitized by NO. These results support strongly the proposal that NO, when sensitizing an active cell, is acting on physiological mechanisms.

The action of nitric oxide in sensitizing the very dry spore is not explainable at this time. A suggestion was made several years ago that the apparent equivalence of nitric oxide and of oxygen in sensitizing the wet metabolizing cell might be due to their similarities in molecular structure. Each has unpaired orbital electrons, and they may accelerate certain kinds of energy shifts and transitions in other molecules. This purely physical explanation of its action should apply especially well in inert systems, but the fact is that in the wet inert system NO is not potentiating at all (it probably is protective), even though a definite O_2 effect is still observed (Figure 11 at 22 mm). There is a marked difference in their actions, with the potentiating effect of NO being completely suppressed at saturation. It is only in the metabolizing system that NO is similar to O_2 at these water levels. In the very dry spore, on the other hand, the sensitizing effect of nitric oxide is almost the same in magnitude as the effect brought about by oxygen when present during irradiation. The "energy-transition" explanation may be entertained for the similarity in actions in this kind of organism.

These observations point up again the value of using biological organisms from which water can be removed in experiments whose purpose is the separation of the physical, chemical, and physiological aspects of radiation damage.

REFERENCES

1. Howard-Flanders, P. Effect of nitric oxide on the radiosensitivity of bacteria. *Nature* **180**, 1191-1192 (1957).
2. Gray, L. H., F. O. Green and C. A. Hawes. Effect of nitric oxide on the radiosensitivity of tumour cells. *Nature* **182**, 952-953 (1958).
3. Sparrman, B., L. Ehrenberg and A. Ehrenberg. Scavenging of free radicals and radiation protection by nitric oxide in plant seeds. *Acta Chem. Scand.* **13**, 199-200 (1959).
4. Powers, E. L., R. B. Webb and B. F. Kaleta. Oxygen and nitric oxide as modifiers of radiation injury in spores of *Bacillus megaterium*. *Proc. Natl. Acad. Sci. U.S.A.* **46**, 984-992 (1960).
5. Dale, W. M., J. V. Davies and C. Russell. Nitric oxide as a modifier of radiation effects on *Shigella flexneri*. *Intern. J. Radiation Biol.* **4**, 1-13 (1961).
6. Petrusek, R. The effects of NO on X-irradiated respiratory sufficient and respiratory deficient strains of *Saccharomyces cerevisiae*. Thesis, University of Texas, Austin, 1964.

WATER AND RADIATION SENSITIVITY

E. L. Powers

PURPOSE AND METHODS

Our investigations on the participation of water in radiation damage in living cells are concerned with two general kinds of experiments: 1) those involving changes in the amount of water in the cell, and 2) those involving changes in the kind of water in the cell. The first phase of the studies in the bacterial spore on the effect of changing the amount of water in the cell was completed some time ago.⁽¹⁾ The first part of our experiments in changing the kind of water in the cell has been brought to completion and is reported herein.

For a number of years, the routine has been to measure radiation sensitivity of bacterial spores produced from vegetative cells that have been grown in H_2O (called " H_2O spores" in this report) and, at the same time, spores produced from vegetative cells grown in D_2O (" D_2O spores"). In addition, there are numerous experiments accomplished in which the amount of different kinds of water (H_2O or D_2O) has been varied in the two different kinds of spores (those grown in H_2O and those grown in D_2O).

The D_2O spores were produced under conditions which make highly likely the complete replacement of the H_2O water by D_2O water, as well as the replacement of certain H atoms by D atoms in such groupings as $-SH$, $-NH_2$, $-COOH$, and the like. Vegetative cells were grown for 8 to 10 generations in 99.9% D_2O together with the usual protiated organic nutrients. The spores derived from these cells probably do not contain deuterium in $-CH$ positions, but certainly do in all water compartments and in easily exchangeable positions.

The methods of equilibrating cells after drying were those employed by Tallentire and Powers. When D_2O was used as the equilibrating gas, allowance had to be made for differences between D_2O and H_2O with respect to their vapor pressures at any given temperature.

PROGRESS REPORT

1) At any given water content, D_2O spores are always less sensitive than H_2O spores. This is true at all temperatures tested, so that the action of D_2O in decreasing radiation sensitivity, when incorporated into the structure of the spore, is not temperature dependent over a wide range of temperature ($5^\circ K$ to $300^\circ K$).

2) When given as the equilibrating gas to D_2O spores, D_2O exerts the following effects: there is invariance in Class I damage at equilibrium vapor pressures below about 0.1 torr; in very dry spores, the free radical component is large; and in very dry spores, the immediate

oxygen effect is large. In the very wet condition, the immediate oxygen effect is considerably smaller, the long-lived free radical component disappears, and the oxygen independent (Class I) damage increases slightly. These are the results reported by Tallentire and Powers for H_2O spores given H_2O .

3) The same general effects are observed when D_2O is the equilibrating gas for H_2O spores; there are, however, interesting quantitative aspects which are noted below.

4) Comparison of H_2O spores with D_2O spores in the very dry state shows that whether H_2O or D_2O is used as the equilibrating gas, the D_2O spores are always less sensitive than the H_2O spores. Furthermore, the absolute difference in sensitivity does not change for that damage independent of oxygen, but this difference becomes larger for both the long-lived free radical component and for the immediate oxygen component as the cells become very dry. Restated: the magnitude of the difference in sensitivity between the H_2O spores and the D_2O spores becomes larger as the absolute amount of water in the cell decreases for both Class II and Class III kinds of damage, but remains constant for Class I kind of damage.

To this point, we have demonstrated that dry cells that have been grown in D_2O show less radiation sensitivity under all conditions than do cells grown in H_2O , and that the difference becomes larger for the free radical and the immediate oxygen components of damage as the cells become very dry.

5) The change in the deuterium effect observed when one changes the water of the cell in the dry region is not associated with the kind of water one adds to or takes away from the cell, but only with the absolute amount. Thus, it makes no difference to the dry spore whether H_2O or D_2O is used as the equilibrating gas. This is a very curious interaction and noninteraction of two kinds of water in the cell. When it is operating as the additive water, no difference is observed between H_2O and D_2O ; yet, both cause a decrease in the difference between the spore produced in H_2O and that produced in D_2O .

6) In regions of wetness (above approximately 0.1 torr), the absolute difference between the H_2O spores and the D_2O spores decreases to a small value. So, the effect of added water throughout the moisture scale is to decrease the differences in radiation sensitivity between the H_2O spore and the D_2O spore.

7) A remarkable difference is made in the region

of wetness by the addition of D₂O in place of H₂O, in contrast to the situation in the regions of dryness where the kind of water makes little or no difference. The spore is always more sensitive when given D₂O than it is when given H₂O, the generalization standing that the H₂O spore is always more sensitive than the D₂O spore under similar experimental conditions.

Therefore, throughout the moisture range the D₂O spores are more sensitive than the H₂O spores when treated alike. In the region of dryness, the added water can be H₂O or D₂O with no experimental difference. In

problems 250 kvp X-rays were used in these experiments). The points to be noted are as follows:

1) As in the instances described above, H₂O spores are always more sensitive than D₂O spores. Compare the top four boxes with the corresponding bottom four boxes from left to right. The incorporation of D₂O into the structure of the spore causes a change in the basic radiation sensitivity seen in the H₂O spore.

2) On the other hand, when H₂O is the suspending medium, the sensitivity observed is always less than that observed when D₂O is the suspending medium for

TABLE 7
INACTIVATION CONSTANTS OBSERVED IN TWO KINDS OF SPORES WHEN SUSPENDED DURING IRRADIATION
IN EITHER PURE H₂O OR D₂O, IN THE PRESENCE AND ABSENCE OF O₂*

SPORES	MEDIUM							
	H ₂ O				D ₂ O			
	O ₂		N ₂		O ₂		N ₂	
	k	S.E. × 10 ⁴	k	S.E. × 10 ⁴	k	S.E. × 10 ⁴	k	S.E. × 10 ⁴
H ₂ O	0.02221	4.1	0.01139	3.0	0.02540	4.3	0.01130	1.8
	0.02355	2.7	0.01100	1.4	0.02454	2.7	0.01239	2.6
	0.02383	2.6	0.01110	1.2	0.02785	3.8	0.01248	6.1
	0.02212	7.1	0.01157	2.3	0.02702	3.2	0.01193	3.4
	0.02853	4.5	0.01055	1.7	0.02488	2.4	0.00974	2.4
	0.02589	3.5	0.01100	0.95	0.02868	6.9	0.01189	0.96
					0.02624	3.2		
	0.0244 ± 0.0004		0.0111 ± 0.0002		0.0263 ± 0.0004		0.0116 ± 0.0003	
D ₂ O	0.01849	1.2	0.00990	1.2	0.02010	2.6	0.01032	1.2
	0.01854	1.7	0.00932	0.84	0.01922	2.6	0.01036	1.7
	0.01798	3.0	0.01023	3.7	0.01864	1.9	0.00987	1.3
	0.01972	3.0	0.01023	0.91	0.02004	2.1	0.00975	1.2
	0.01917	1.5	0.01053	1.5	0.01952	1.3	0.01047	0.89
			0.01037	1.7			0.01045	1.1
			0.01020	1.8			0.01016	0.65
	0.0188 ± 0.0001		0.01009 ± 0.0002		0.0195 ± 0.0002		0.0102 ± 0.0001	

* The numbers are constants from individual experiments; the lowest number in each box is the mean and the standard error of experimental values in the box.

the region of wetness, on the contrary, the spores (whether H₂O or D₂O) given D₂O are always more sensitive than the spores (whether H₂O or D₂O) given H₂O.

In order to establish these surprising relationships more carefully, we made a series of measurements of the sensitivity of spores at the ultimate in wetness—in aqueous suspension. Table 7 is a summary of these experiments. As usual, we report inactivation constants as measures of sensitivity. The table reports sensitivity of spores grown in H₂O and those grown in D₂O when H₂O is used as a suspending medium, and when D₂O is used as a suspending medium, under conditions of anoxia and oxygen presence (because of penetrance

similar experiments. To observe this, we compare the top box number 1 with the top box number 3, or the top box number 2 with the top box number 4 to see that this obtains for H₂O spores. It also can be seen in D₂O spores if one compares bottom 1 with 3, or bottom 2 with 4.

The differences observed in some of these pairs are very small. But direction-wise, the statements made are correct; and the fact that under conditions of less moisture these differences become larger reinforces our belief that these differences, though small, are real.

Therefore, at least three levels of water organization are indicated by these experiments, as recognized in terms of radiation sensitivity. They are as follows:

1) There is "structural" water that we do not interfere with in our ordinary pumping and drying procedures. When D₂O occupies this water space, the cell is always less radiation sensitive than it is when H₂O occupies this water space. The magnitude of the difference is different for different amounts of water.

2) There is a water compartment affecting radiation sensitivity that can be occupied either by H₂O or by D₂O. What is important is not the kind but the amount of water in this compartment. It is curious that the difference in radiation sensitivities of the H₂O spore and the D₂O spore changes when the amount of water changes, but the change can be accomplished with either H₂O or D₂O as the additive.

3) The third water compartment is observed at very high water levels. Water added as D₂O results in sensitivities that are higher than those observed when H₂O is added.

These results are considered starting points for in-

vestigations explaining the action of water in radiation sensitivity. Now we have reason to believe that by looking at the differences in the physical and chemical aspects of the interaction between radiation and H₂O on one hand, and radiation and D₂O on the other, and correlation of these with our biological results, we can find some evidence bearing on mechanisms of damage in cells.

Note: The work described in this brief report is illustrated fully in the paper, "Some physico-chemical bases of radiation sensitivity in cells," in *Cellular Radiation Biology*, Proceedings of the 18th Annual Symposium on Fundamental Cancer Research, M. D. Anderson Hospital, Houston, Texas (in press).

REFERENCE

1. Tallentire, A. and E. L. Powers. Modification of sensitivity to X-irradiation by water in *Bacillus megaterium*. *Radiation Res.* **20**, 270-287 (1963).

PROPERTIES OF SURVIVORS AFTER X-IRRADIATION OF SINGLE CHINESE HAMSTER CELLS IN CULTURE

Warren K. Sinclair

PURPOSE AND METHODS

Single-cell culture techniques with established lines of mammalian cells enable several features of radiation response to be examined which cannot be observed *in vivo*. One such aspect is the variation in properties among the survivors of a given dose of X-radiation. It has been quite widely observed, though not previously studied, that while the colonies formed by single mammalian cells may be quite uniform on unirradiated plates, they vary greatly in size on heavily irradiated plates. The spread in colony size increases with the dose. Such colonies evidently contain a population of cells (derived from a single irradiated cell) which has sustained a form of sublethal damage which has persisted among the cells of the colony at least until the time of scoring. The object of this study is to examine the properties of the subline populations developed from each individual surviving clone and if the damage persists, to try to determine its nature.

Single cells of the V79 near-diploid Chinese hamster line, inoculated on to plastic Petri dishes, are exposed to a given dose of 250 kvp X-radiation, hvl = 0.9 mm Cu, dose rate 110 R/min (absorbed dose 0.945 rad/R). Surviving cells incubated at 37°C in HUT-15 liquid medium, in a humid atmosphere of 2% CO₂ and air grow to visible colonies which are scored after ~12 days. After a dose of 1500 R (1420 rad) colonies are selected from the spectrum of sizes available on the plate, each is trypsinized independently, and new sub-

lines are developed from each clone. The clones selected include *large colonies*, 3-4 mm in diameter, 20,000-100,000 cells per colony; *medium colonies*, 1-2 mm in diameter, 1000-5000 cells per colony; *small colonies*, less than ~0.5 mm, 40-200 cells per colony. After 1500 R at least 40% of the colonies are small.⁽¹⁾ None of the colonies selected failed to grow.

The subline populations thus developed are then investigated with regard to:

- a) growth and cell size distribution
- b) plating efficiency
- c) radiation survival
- d) chromosome composition (ploidy)
- e) chromosome aberrations

PROGRESS REPORT

Many colonies (more than 20) of the large and of the small type following 1500 R have been isolated and the properties of their progeny populations determined. A few medium size colonies have also been studied.

Properties of Surviving Colonies

The principal features characterizing the different types of colonies are

- a) Large colonies: grow rapidly [doubling time (D.T.) = 10-15 hr] and plate well [plating efficiency (P.E.) 60-90%]; and these and their radiation survival properties [the extrapolation number when the exponential part of the curve meets the zero dose axis (*n*) ~4; the reciprocal slope of the exponential part of the survival curve (*D*₀) ~200 rad] are also characteristic of the V79 parental line.

However, these colonies exhibit changes in cell size distribution correlated with changes in ploidy (40–50% of the large clones are tetraploid after 1500 R) and for some time after exposure maintain chromosome aberrations of various kinds.⁽²⁾

- b) Small colonies: grow slowly (D.T. = 20–35 hr), plate poorly (P.E. 20–40%) and do not survive subsequent X-radiation as well as large colonies or the parental line ($n \sim 4$, $D_0 \sim 140$ rad). They contain chromosome aberrations and ploidy changes similar, at least grossly, to those observed in large colonies.⁽²⁾ The radiosensitivity is, however, characteristic of the small colony and not of the ploidy of either large or small colonies. (See Figure 12 and Reference 1.) Small colony cells also exhibit some distinct morphological features; cells are more granulated, some are spindly and elongated, and most attach more firmly to glass or plastic surfaces, than do cells of the parental line.
- c) Medium colonies: grow with intermediate properties but have either normal radiosensitivity or the greater sensitivity of the small colonies, not “in-between” values. Only a few studies have been made of this type of colony however.

Persistence of the Small-Colony Type

The cells of small colonies appear to recover close to normal properties in a period sometimes as short as 1–3 months. However, some of the sublines isolated have maintained their original characteristics for as much as 3 years. In this period, the plating efficiency and growth may improve (presumably by selection at transfer), but the radiation properties remain substantially unaffected. This damage is evidently heritable over long periods and may even persist indefinitely.

Isolation of Unirradiated Small Colony

Since few phenomena induced by radiation do not occur spontaneously, the small-colony type of damage was sought among unirradiated cells. After a series of successive selections, a small-colony population was isolated from the unirradiated parent line and shown to exhibit properties similar to the irradiated small colonies.⁽³⁾

Small-Colony Generation Time and Viability

Recent measurements on one small-colony subline (carried out with Jalane Christensen) by time lapse photomicrography showed a mean generation time of 22.2 ± 0.9 hr compared with 10.3 ± 0.2 hr for the normal V79 line. The percentage of viable progeny for cells that plate out was found to be 100% for the parental line and about 90% for the subline (i.e., 10% of the subline cells were inviable).

Labeling with H^3 -thymidine in both lines has shown that the generation cycle of the subline is lengthened at all stages by about a factor of 2, i.e., the S period increased from about 6 to 12 hours.

Further chromosome studies on colonies isolated after 1500 R (Dr. C. K. Yu) are now in progress.

CONCLUSIONS

Small-colony cells surviving 1500 R of X-radiation and induced readily at that dose (40% or more of the colonies are of the small type) also occur, spontaneously, but much less frequently. Populations of these cells are slow growing, plate poorly and have a greater radiosensitivity than large colony survivors. The damage expressed in these properties is heritable and may persist indefinitely. Its origin is uncertain, but since both large and small colonies exhibit similar changes in chromosome composition and aberrations, this damage

FIG. 12.—X-ray survival of large colonies (Experiment 71, near-diploid; Experiment 72, near-tetraploid) and small colonies (Experiment 79, near-diploid; Experiment 80, near-tetraploid).

does not seem to be associated with observable gross changes in the chromosome apparatus. The damage may be more general in nature (i.e., both cytoplasmic and nuclear) or it may be transmitted via specific cellular material other than the chromosomes.

REFERENCES

1. Sinclair, W. K. X-ray induced heritable damage (small-colony formation) in cultured mammalian cells. *Radiation Res.* 21, 584–611 (1964).
2. Sinclair, W. K. and A. N. Stroud. Chromosome numbers and aberrations in large and small clones of irradiated Chinese hamster cells in culture. Argonne National Laboratory Biological and Medical Research Division Semiannual Report, July through December 1962. ANL-6790, pp. 161–170.
3. Sinclair, W. K. and Carolan Wieker. Spontaneous nonlethal damage in a small clone of Chinese hamster cells in culture. Argonne National Laboratory Biological and Medical Research Division Semiannual Report, July through December 1962. ANL-6790, pp. 156–160.

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

Warren K. Sinclair and Richard A. Morton

PURPOSE AND METHODS

Biochemical events are presumed to occur sequentially within the generation cycle of the mammalian cell, i.e., with the increasing age of the cell between one division and the next. Consequently, it is possible that the radiosensitivity of cells varies with the age within the cycle and may be related to events known to occur within the cell as a function of time. Both ionizing radiation and ultraviolet radiation may be useful for studying these events.

Although different techniques of labeling in asynchronous populations have enabled some phases of the generation cycle to be defined, this type of study is much more effectively performed with synchronized cells, preferably commencing with cells all at zero age and retaining these cells in reasonable synchrony for at least one and preferably more cycles.

This synchronized population may then be used to study survival (and other radiation responses) by the single-cell cloning technique as a function of age of the cell.

PROGRESS REPORT

Asynchronous Population

Cells of a near-diploid Chinese hamster subline V79 are normally grown on plastic Petri dishes in liquid HUT-15 medium in a humid atmosphere of 2% CO₂ and air at 37° C. The doubling time in log phase is 10 hr, and studies by both time lapse photomicrography and labeling with H³-thymidine (carried out with C. Wieker and J. Christensen) have established that cells that plate out are 100% viable. Also, the generation time, 10 hr, comprises, first a G₁ period of 1.5 hr, then an S period of 6.0 hr, followed by a G₂ period of about 1.5 hr and a mitotic phase of about 1.0 hr. The survival response to X-rays (250 kvp, 0.9 mm Cu hvl, 110 R/min, 0.945 rad/R), determined by plating single cells and scoring visible colonies after about 12 days, fits reasonably well the relation

$$s = 1 - (1 - e^{-D/D_0})^n \quad (1)$$

where s is the surviving fraction and D the dose, with $n \sim 4$ and $D_0 \sim 200$ rad.⁽¹⁾

Synchronized Population

The synchronized population is obtained by a combination of cold shock and gentle agitation which selects for cells at or close to division.⁽²⁾ The technique is con-

sistent and reproducible. It provides a limited population of cells which are quite well synchronized but includes in addition to cells about to divide (mother cells) cells just divided (daughter cells); consequently, the mitotic index is not the best index of synchrony. Furthermore, in the first generation cycle, mother cells form 2-cell units while daughters remain as a 1-cell unit.

Degree of Synchrony

The starting population desynchronizes with time, and measures of synchrony vary according to the stage at which they are applied. Several methods have been used⁽³⁾ and these include the initial mitotic index (often 50% or more), growth (per cent phasing index is $\sim 70\%$), and labeling with H³-thymidine (labeling index, the difference between maximum and minimum percentage of cells labeled, is often $\sim 70\%$ or more).

X-Ray Response

The variation of survival (scored by ability to form colonies) is shown in Figure 13. Typically the survival is low in G₁, rises during the latter part of S (at about 10 hr) and falls again during G₂. The curve labeled f is the observed colony response, while curve s is the response for single cells derived from the data for f using⁽³⁾

$$s = 1 - (1 - f)^{1/N} \quad (2)$$

Curve s shows the expected desynchronization in the second cycle. The form of this response is not altered markedly over a wide range of dose levels.

Survival curves over more than two decades (Figure 14) show that the resistance observed in the latter part of S (Figure 13) is mainly due to the increase in the shoulder of the curve in S (i.e., at 10 hr). Figure 14 shows the data for f (the colony survival), but s , the single-cell survival, may be derived from it using Equation 2. When this is done s fits Equation 1 reasonably well for G₁ and G₂ cells with $n \sim 2$, $D_0 \sim 200$ rads, while for S cells n is ~ 8 , $D_0 \sim 200$ rads, but the fit is poor and there may also be a change of slope in this phase.

In fact, cells in G₁ and G₂ are difficult to obtain entirely free of a few S cells, and since these are more resistant, the true response of G₁ and G₂ cells may have a slightly smaller shoulder than that observed here. This point is being investigated further by destroying contaminating S cells by the incorporation of lethal amounts of H³-thymidine.

Ultraviolet Exposure

Similar survival experiments carried out using ultraviolet radiation from a germicidal lamp (but removing medium at the time of exposure) show a different type of response. Survival curves after ultraviolet radiation indicate that while the extrapolation number varies similarly to that found for X-rays and is greatest in S, the slope also changes markedly and is greatest in S, ($D_0 = 34$ ergs/min²), less in G₁, ($D_0 = 39$ ergs/min²) and much less still in G₂ ($D_0 = 72$ ergs/min²). Thus depending to some extent on the dose level, G₂ is usually the most resistant phase.

Both ultraviolet and X-ray age responses correlate quite well with the response calculated for and experimentally determined for asynchronous populations.⁽³⁾

FIG. 13.—Survival of synchronized V79 hamster cells after a single dose (710 rad) of 250 kvp X-radiation against time after inoculation (i.e., age response). Curve *f* represents the observed colony surviving fraction and curve *s* the single cell age response calculated according to $s = 1 - (1 - f)^{1/\bar{N}}$ where $\bar{N}$ is the mean cellular multiplicity at any given time (see text).

CONCLUSIONS

Synchronized populations of Chinese hamster cells demonstrate clearly the variation of both X-ray and ultraviolet survival response with age. X-ray resistance (primarily a change in shoulder) is greatest in the latter part of the S period. Thus, for reasons which are not as yet resolved, S cells can withstand greater amounts of

sublethal damage due to X-rays than cells in G₁ or G₂. This seems also to be true for ultraviolet radiation except that additionally the slope of the survival curve is modified and is so reduced in G₂ that this period is more resistant than S.

FIG. 14.—Survival curves for synchronized V79 hamster cells exposed to graded doses of 250 kvp X-radiation at these different times during the first generation cycle after inoculation. Curve 1, 2.2 hours (G₁ cells); Curve 2, 10.4 hours (S cells); Curve 3, 15.3 hours (G₂ cells).

The data shown are the observed colony survivals (*f*). The response for single cells (*s*) could be found by applying $s = 1 - (1 - f)^{1/\bar{N}}$ with $\bar{N} = 1.82$, which modifies the extrapolation numbers to 2 for curves 1 and 3 and 8 for curve 2. The reciprocal slope D_0 is about 200 rads in all cases, but the fit of the data to $s = 1 - (1 - e^{-D/D_0})^{1/\bar{N}}$ is only a rough first approximation in the case of cells in the S phase (10.4 hr).⁽³⁾

Understanding of the relation of these responses to specific biochemical processes is primarily limited at present by inadequate knowledge of the detailed time sequence of events within the generation cycle. Synchronized cells, especially if available in large enough quantity, may be invaluable for the study of these events.

REFERENCES

1. Sinclair, W. K. X-ray induced heritable damage (small colony formation) in cultured mammalian cells. *Radiation Res.* **21**, 584-611 (1964).
2. Sinclair, W. K. and R. A. Morton. Variations in X-ray response during the division cycle of partially synchronized Chinese hamster cells in culture. *Nature* **199**, 1158-1160 (1963).
3. Sinclair, W. K. and R. A. Morton. X-ray and ultraviolet sensitivity of synchronized Chinese hamster cells at various stages of the cell cycle. *Biophys. J.*, **5**, 1-25 (1965).

REPAIR OF X-RAY DAMAGE IN SYNCHRONIZED MAMMALIAN CELLS IN CULTURE

Warren K. Sinclair and Richard A. Morton

PURPOSE AND METHODS

The process of repair after X-ray damage in mammalian cells has been investigated in asynchronous populations by the method of two doses separated by varying time intervals between them.⁽¹⁾ Since the X-ray response of cells varies with age in the generation cycle (see previous report), the kinetics of survival after the first dose and, therefore, the response to the second dose are complicated in asynchronous populations by the presence of different age (and sensitivity) states and subsequent progression through the cycle.^(2, 3) If a synchronized system is used, the effects of cell progression and sensitivity states can be defined and the process of repair investigated free of such encumbrances. Furthermore, it is possible to investigate the magnitude of repair at different cell ages and perhaps eventually relate it to specific biochemical processes occurring in the cell.

The method of synchronizing Chinese hamster cells described in the previous report is used to provide a population of cells quite well synchronized at about zero age. These cells are followed throughout several generation cycles. In the first cycle, the first of two equal doses can be given at different cell ages and the survival of these examined by the single-cell cloning method following a second dose given at intervals. The second dose may also be varied in magnitude to provide survival curves over a range of second doses.

PROGRESS REPORT

Recovery for Cells in S

To demonstrate the existence of recovery free of cell progression through the cycle, we chose to give the first dose (660 rad) at that point in the cycle where X-ray resistance is a maximum (i.e., late in S, at about 10 hr after inoculation). Second doses of 660 rad were then given at intervals of time thereafter—during this time surviving cells can only progress, if they do at all, to a more sensitive state. However, as shown very clearly in Figure 15, Curve C, the survival rises rapidly as the interval between doses increases, to a maximum (a factor of 3 or 4 in survival) when the interval is about 2.5 hr. Therefore, Curve C follows the same general pattern as Curve B (1320 rad, single dose) but is separated from it in time (due to division delay) and in survival level, being constantly at a higher level throughout. This establishes that repair definitely occurs, it is rapid and may have reached its maximum value by 2–3 hr but certainly it appears to have done so by the next

division. Labeling studies show that irradiated cells continue to progress slowly through the cycle.

Magnitude of Recovery in S

The magnitude of recovery in S can be assessed by comparing the single-cell response to a first dose of 660 rad (Curve A, Figure 16) with the single-cell response to a second 660 rad of survivors of the first 660 rad (Curve

FIG. 15.—Two-dose response of synchronized V79 hamster cells given first dose at their most resistant stage in the first generation cycle. Curve A, single dose response for 660 rad; Curve B, single dose response for 1320 rad; Curve C, two-dose response for cells of age 10.6 hr given 660 rad, restored to 37° C for different lengths of time, then given a second 660 rad.

B, Figure 16). This requires some analytical manipulation,⁽²⁾ but the result is clear. After about 2.5 hr and certainly by the next division the response to the second dose is just like that to the first (but displaced in time due to delay). Evidently, insofar as colony formation is concerned, the damage is fully repaired by the next division after exposure.

Recovery at Other Stages of the Cycle

Experiments were carried out with first doses at various cell ages—2.5 hr (G_1), 7.0 hr (early S), 10.6 hr

FIG. 16.—Calculated age-response of single synchronized hamster cells to 660 rad. Curve A, previously unirradiated cells; Curve B, cells which survived a first dose of 660 rad at 10 hr.

(late S), 13.0 hr (G_2). In each case, the response to a second dose indicated that recovery occurred at all cell ages. This may, however, not be definitely established for true G_1 and G_2 cells because of the presence of a few S cells in the population at the time, the proportion of which will be increased after the first dose. Experiments are now being undertaken to remove these S cells by incorporating lethal amounts of H^3 -thymidine.

Estimated Response of Asynchronous Populations

In spite of the limitations on our knowledge concerning G_1 and G_2 cells, the responses at the four cell ages mentioned above can be compounded to yield the expected response for the distribution of cell ages to be found in an asynchronous population. This requires estimating the response to the second dose for each of the four situations and applying this to the distribution in age of the survivors of the first dose.⁽²⁾ The result is shown in Figure 17. Curves I, II, III, and IV represent respectively the contribution of cells of age (0–5 hr), (5–9 hr), (9–12 hr), and (12–16 hr) to the asynchronous population and their sum is Curve A, the estimated response to two doses of 660 rad separated by varying intervals of time. Curve B shows actual experimental data for an asynchronous population. Curves A and B are quite similar in form and Curves I–IV show how the cells of various ages contribute in different ways to the over-all response. Thus, the maxima in Curves A and B are mainly due to S cells, while the minima have G_1 cells (progressed in time of course) as a principal contributor.

FIG. 17.—The response of an asynchronous population of single cells to two doses of 660 rad separated by different time intervals. Curve A, estimated from the sum of four components, I, II, III, IV receiving first doses at different cell ages, 2.5, 7.0, 10.6, and 13.0 hr respectively. Curve B, experimental data for an asynchronous population exposed to 730 rad plus 730 rad. Curve B had the same survival level as Curve A at the peak response, that is, at an interval of about 2.5 hr between doses.

Second-Dose Survival Curves

Presumably the repair process is associated with the shoulder of the survival curve. To examine this, cells at the maximum resistance (i.e., in S) were given a first dose of 660 rad, allowed to reach the maximum point in the recovery curve (curve C, Figure 15), (i.e., 2.3 hr after the first dose) and then given a series of graded second doses. A survival curve resulted in which it was apparent that a large part of the shoulder had returned and the slope (as for most S cells) was somewhat steeper. A similar procedure was followed to give graded second doses at the minimum point in the recovery curve (i.e., 7.1 hr after the first dose). This survival curve had a much smaller shoulder and a somewhat smaller slope, characteristic of cells which had reached G₂ or G₁.

Consequently over a wide range of doses cells appeared to recover their former properties quickly.

CONCLUSIONS

Experiments with the synchronized hamster populations have established the existence of a repair process free of complications from cell progression through different age (and therefore sensitivity) states. They have also shown that this process is rapid and may be almost complete by 2-3 hr and is certainly so (insofar as colony formation is concerned) by the time of the next division.

PHOTODYNAMIC MUTAGENESIS

Robert B. Webb

PURPOSE AND METHODS

Visible light may have striking effects on cells that have absorbed small amounts of certain dyes and other agents. These responses include cell lysis, loss of some metabolic capability, loss of capacity to divide, chromosomal aberrations, and mutations. Analogous effects have also been observed in molecular *in vitro* systems. These photosensitized effects involve transfer of energy from the dye molecule absorbing the radiant energy to sensitive molecules in the cell. Oxygen is usually involved in the process. The photodynamic process should be useful in studying the primary process of mutation. For in the presence of light, acridine orange is a very effective mutagen under conditions yielding no observable cell death.⁽¹⁾

The purpose of this study is to obtain information bearing on energy transfer processes within cells and on basic biological processes of gene replication, mutation, and cell reproduction.

Different agents have been examined for effects on mutation rates in continuous cultures. Continuous cul-

The kinetics of the process can, in fact, be studied in greater detail with this system.

These facts seem to be true over a wide range of second doses, and it is also clear that the recovery process is intimately associated with the return of a shoulder in the survival curve.

Two-dose experiments at different cell ages, while they do not yet prove the existence of recovery in G₁ and G₂ cells, certainly suggest it and in any event provide considerable information on the fine structure of the kinetics of asynchronous populations following fractionated doses.

Linking repair phenomena with biochemical processes requires much further study, but populations of synchronized cells will undoubtedly play a considerable part in the eventual clarification of such relationships.

REFERENCES

1. Elkind, M. M. and H. Sutton. Response of mammalian cells grown in culture. I. Repair of X-ray damage in surviving Chinese hamster cells. *Radiation Res.* **13**, 556-593 (1960).
2. Sinclair, W. K. and R. A. Morton. Recovery following X-irradiation of synchronized Chinese hamster cells. *Nature* **203**, 247-250 (1964).
3. Sinclair, W. K. and R. A. Morton. Survival and recovery in X-irradiated synchronized Chinese hamster cells. *Cellular Radiation Biology*, Proc. 18th Ann. Symp. on Fundamental Cancer Research, M. D. Anderson Hospital, Houston, Texas, 1964. University of Texas Press, in press.

tures of *Escherichia coli* B/r/1,try⁻ were grown in a minimal medium containing tryptophan and limited with glucose or tryptophan. Mutation to T5 resistance was assayed by methods previously described.⁽²⁾ Dyes were added in concentrations from 5×10^{-7} M to 10^{-5} M. For dark experiments the chemostats were placed in a light shield. In other experiments, light was supplied by a 4-watt, white fluorescent lamp placed from 6 to 20 cm from the growth tube. Light intensities were measured with a Westinghouse illumination meter.

PROGRESS REPORT

The effects of acridine orange (AO), acridine yellow, proflavine, atebrin and methylene blue on rate of mutation to resistance to the bacteriophage T5 have been examined. In the presence of light and oxygen all of these dyes except atebrin are effective mutagens under conditions yielding little or no cell death. Atebrin, which may slightly increase the mutation rate in the dark, showed no additional effect in the light under conditions used. Photodynamic mutagenesis is char-

acterized by a surprisingly short mean delay in expression of phage resistance, a recessive character. The mean delay is about 1.5 e-fold generations instead of the value of 3.4 e-fold generations reported by other workers⁽²⁾ for this mutation when induced by caffeine or ultraviolet light.

Although dosimetric means available do not permit precise statements of the relative efficiencies of the agents in inducing mutation, a tentative qualitative comparison can be made. For the conditions used, the order of effectiveness appears to be 1) acridine yellow, 2) proflavine, 3) acridine orange, and 4) methylene blue.

For AO, within the range of experimental error, mutation rate is proportional to dye concentration over the range of 10^{-6} M to 10^{-5} M. Mutation rate is proportional to light intensity for over-all intensity values of 75 foot-candles to 550 foot-candles.

PHYSICAL AND PHYSIOLOGICAL COMPONENTS OF RADIATION DAMAGE IN CELLS

Robert B. Webb

PURPOSE AND METHODS

It has been shown by many workers that radiation effects can be greatly modified through metabolic processes. Metabolic involvement is at least a strong possibility in most biological responses to radiation. It is, therefore, necessary to design experiments with the purpose of separating physical and chemical events from metabolic processes. The use of *in vitro* systems of biological origin has failed to provide answers to important biological questions since the end point in the experiments is usually not biological in nature. An important separation of physical and chemical events from metabolic processes in the irradiated cell has been achieved in the bacterial endospore by Powers and co-workers.⁽¹⁾ The spore contains the complete genetic information and has all necessary cytoplasmic components required to produce a fully functioning vegetative cell; however, the endospore is completely dormant, even in the fully hydrated state, until germination is induced. Modification of the radiation response in the dormant spore by physical and chemical treatments before the onset of germination almost certainly affects chemical and physical events rather than metabolic processes.

These studies were initiated with the purpose of extending the observations made on the bacterial spore to metabolically capable cells.

Recently it has been possible to maintain high viability in vegetative bacteria desiccated after being washed

CONCLUSIONS

The observation that the mean delay of expression for T5 resistance is much shorter for photodynamic induction than for caffeine or ultraviolet light induction suggests that the initial alteration produced by these photodynamic agents may be closer to the fundamental alteration in the genetic material than is the case with either caffeine or ultraviolet light.

For acridine orange in the absence of light, mutation rates are significantly lower than spontaneous mutation rates. In the dark acridine orange seems to be antimutagenic.⁽¹⁾

REFERENCES

1. Webb, R. B. and H. E. Kubitschek. Mutagenic and antimutagenic effects of acridine orange in *Escherichia coli*. *Biochem. Biophys. Res. Commun.* **13**, 90-94 (1963).
2. Kubitschek, H. E. and H. E. Bendigkeit. Latent mutants in chemostats. *Genetics* **46**, 105-123 (1961).

with distilled water. This technique makes possible the study of processes in the irradiated cell in the absence of both water and metabolism. Vegetative cells are suspended in distilled water, mounted on membrane filters, dried slowly under anoxic conditions, without freezing, to a pressure of about 5 torr, then pumped to low pressures. Cells dried in this way remain viable for several days if oxygen is excluded. The dry cells are slowly inactivated in the presence of oxygen. The rate of this inactivation increases sharply as the degree of hydration decreases. Water content is controlled by the method of Tallentire and Powers.⁽²⁾

The X-ray dose-survival response is the primary experimental technique employed. These techniques have been previously discussed.^(1, 2)

PROGRESS REPORT

Recent studies with *Staphylococcus aureus* FIDA 209 dried on membrane filters indicate that under controlled hydration levels below 5 torr, the general pattern of radiation sensitivity in this vegetative system is like that previously observed in the bacterial spore.⁽²⁾ One general observation is that, in the presence of oxygen, drying increases the over-all X-ray sensitivity of these cells. Furthermore, this increase is in the oxygen-dependent components of X-ray damage. The oxygen-independent part of X-ray damage remains almost constant at hydration levels between 5 torr and 10^{-3} torr.

Studies on protection against X-ray damage showed that cells dried in the presence of glucose or cysteine are more resistant than those dried from distilled water. This protection includes both the oxygen-dependent and oxygen-independent parts of radiation damage. This protection was evident at levels of hydration from less than 10^{-3} torr to the water-soaked state. Characteristics of this protection suggest that energy transfer in the solid state may be involved in this action. At hydration levels above 5 torr, the vegetative cell departs substantially from the response of the metabolically dormant spore.⁽²⁾ In this region radiation sensitivity increases by a factor of 2.5 as the level of hydration increases to the water-soaked state both for aerobic and anaerobic conditions. (In the bacterial spore, under aerobic conditions, radiation sensitivity continued to decrease as levels of hydration increased to the water-soaked state, whereas, under anaerobic conditions radiation sensitivity increased slightly as the level of hydration increased to the water-soaked state.⁽²⁾) In the vegetative cells, at the region of hydration between 5 torr and 23 torr where endogenous respiration is measurable, treatments that alter metabolic capability (carbon monoxide, nitric oxide, and water content) greatly alter the response of the cell under both aerobic and anaerobic conditions.

CONCLUSIONS

These studies reveal that the three components of X-ray damage previously identified in the bacterial spore by Powers and co-workers^(1, 2) are also operating in these vegetative cells. These are Class I, independent of oxygen either during or after irradiation; Class II, dependent on the presence of oxygen during irradiation; and Class III, dependent on the presence of oxygen after irradiation. Class III damage has been identified as a free radical component in the vegetative system.

Preliminary studies suggest that the increase in sensitivity observed in the vegetative system between 5 torr and 23 torr under both aerobic and anaerobic conditions may not be caused primarily by radiolytic products of water. An alternate possibility is that the increase in sensitivity is related to an increased physiological capability of the cell at these hydration levels.

These findings support the proposal of Powers and co-workers that mechanisms demonstrated in the bacterial spore operate in other biological systems.

REFERENCES

1. Powers, E. L., R. B. Webb and C. F. Ehret. Storage, transfer, and utilization of energy from X rays in dry bacterial spores. *Radiation Res. Suppl.* 2, 94-121 (1960).
2. Tallentire, A. and E. L. Powers, Modification of sensitivity to X-irradiation by water in *Bacillus megaterium*. *Radiation Res.* 20, 270-287 (1963).

CYTOGENETIC STUDIES WITH IONIZING RADIATION IN MAMMALIAN CELLS IN VITRO

C. K. Yu and Warren K. Sinclair

PURPOSE AND METHODS

Ionizing radiation-induced chromosome abnormalities in cultivated mammalian cells have been known for many years; however, the relationship between radiation dose and the manifestation of genetic injury is still not fully understood. Most laboratories have placed their emphasis on the frequency and type of chromosome aberrations appearing soon after irradiation with a relatively low dose. The objective of this study is to understand the genetic changes induced at higher doses especially with the following interests: 1) polyploidization, 2) chromosome constitution of giant cells, 3) lethality of chromosome aberrations, 4) relationship between dose response and type of aberrations, and 5) chromosome aberrations induced at higher doses.

An established near-diploid cell subline of the Chinese

hamster (*Cricetulus griseus*) with a single karyotypet 122D₁ and also 122T cells are being used for this study. Cells are inoculated into 85-mm plastic Petri dishes, using HUT-15 medium and incubated at 37° C in a humid atmosphere of 2% CO₂ and air. Cells are irradiated at room temperature with 250 kvp X-rays (hvl ~ 0.9 mm Cu) at a dose rate of about 110 R/minute. Five doses are usually used, namely: 0, 250, 500, 1000, and 1500 R (1 R = 0.945 rad). After exposure to X-rays, cells are returned to the incubator. Observations of chromosomes are made on each sample at different intervals after irradiation. Cytological preparation involves colchicinization of cells at a concentration of 2 µg/ml culture medium for 2 hours, hypotonic treatment with 0.9% sodium citrate for 30 minutes, fixation in acetic alcohol (1:3) for 1 hour, air-dry technique, and staining with 2% aceto-orcin.

PROGRESS REPORT

The peak incidence of polyploidy (specifically, in this case, the doubling of the stemline chromosome number) in both diploid and tetraploid cell lines is found at about 48 hours after exposure to X-rays. Both lines had a very similar dose response. Endoreduplication (endomitosis) is found to be the principal cytological mechanism for the formation of polyploidy. There are two types of induced polyploidy, those with the true polyploid complement (number of chromosomes doubled) and those with the diplochromosome configuration of the parental number. The highest yield of diplochromosome cells is found at early hours of assay. The decrease of diplochromosome cells and the increase of true polyploid cells at later hours are probably due to conversion of diplochromosome cells into true polyploid cells after further division.⁽¹⁾

At the present time, the giant cells induced by X-rays are those highly polyploid cells with chromosome numbers up to 64N (more than 700 chromosomes). Giant cells of ploidies of 8N or higher comprise about 80% of the mitotic population between 48 and 144 hours after irradiation. Most of the giant cells observed contain a single nucleus. The cytological mechanism responsible for the formation of giant cells is known as endomitosis.

In a diploid cell line, the appearance of various types of chromosome aberration induced by X-rays depends upon the time of assay after exposure, which also affects the aberration rate. At the optimum time of assay, the percentage of complex aberration is found to

be proportional to the square of the dose. The percentage of simpler aberrations approaches a linear dose-response relationship. In a tetraploid cell line, the chromosome aberrations have been found at a much higher rate than in the diploid cells. Various polycentric chromosomes, such as chromosomes with 14 centromeres, are only induced by X-rays in tetraploid cells.

It has been shown in the literature that cells with severe chromosome damage, e.g., complex aberrations, will definitely die. In our studies, types of chromosome aberrations induced by X-rays at higher doses up to 1500 R are mostly multibreakages, e.g., chromosome fragmentation. Cells with chromosome fragmentation are found with maximum value between 50 and 74 hours after X-irradiation. Thereafter, the number of cells containing fragments diminished. This is apparently due to the death of fragmented cells. The formation of polycentric chromosomes may be due to a union of several chromosome fragments or due to a union of two di-, or trivalent, diplochromosomes as a result of endoreduplication. The latter shows a bilaterally identically symmetric configuration, i.e., their right and left halves are mirror images of each other. Further studies of chromosome aberrations induced by ionizing radiation are being performed.

Studies on the types of aberration induced by X-rays in synchronized cells 122D₁ of the Chinese hamster irradiated at different cell ages are now in progress.

REFERENCE

1. Yu, C. K. and W. K. Sinclair. Polyploidy induced by X-rays in Chinese hamster cells *in vitro*. *Science* **145**, 508-510 (1964).

THE ESTABLISHMENT OF CHROMOSOME HOMOGENEITY IN SOMATIC CELLS IN VITRO

C. K. Yu and Warren K. Sinclair

PURPOSE AND METHODS

Mammalian cells in continuous culture have been found to have remarkable changes in morphology, growth characteristics, and especially chromosome constitution. Because the culturing of cells is an adaptive process, cells *in vitro* never experience the perfect growth conditions of the *in vivo* states. Chromosome aberrations may, and usually do, occur spontaneously and lead the cell population to a quasi-diploid status, with a stemline number of chromosomes. Therefore, the maintenance of a classical diploid cell *in vitro* is almost impossible.

Genetic homogeneity of cultured cells is most important in studies of radiation response. In order to

achieve this, it has been suggested that in a cell population a predominant karyotype that has adapted to the environment could be selected and could be maintained without further chromosome changes. Therefore, if a clone arises from an isolated single cell of this karyotype, the resulting cell population should have only this karyotype and be able to maintain it for a long period. The establishment of such cell sublines is of basic importance in radiation studies.

This study involves a series of processions, namely, selection of chromosome karyotype, culture methods, and cloning techniques. A quasi-diploid cell subline V-79 of Chinese hamster (*Cricetulus griseus*) is used. An extensive survey of the chromosome karyology has been

carried out and completed⁽¹⁾ and a predominant karyotype found.

PROGRESS REPORT

The cloning technique has been used to establish possible cell sublines from clones isolated from single cells. Of course, there is no means of observing the karyotype of a cell before selection. Therefore, a number of clones were produced and subjected to chromosome examination until a cell subline labeled 122D₁ with such a predominant karyotype was found. At the same time, a less dominant diploid karyotype 122D₂ was also isolated as well as a tetraploid subline 122T.⁽²⁾ The cell sublines were established and have since been grown in log phase on HUT-15 medium, at 37° C in a

humid atmosphere of 2% CO₂ and air. Chromosome studies are being carried out, and in 122D₁ no chromosome changes have been found since the cell line was established. However, karyological examination is being continued to see whether or not the cell line will eventually change its genetic homogeneity. 122D₂ changed quite quickly, and 122T also changed after a few months.

REFERENCES

1. Yu, C. K. Evaluation and nature of the near-diploid cells of Chinese hamster (*Cricetulus griseus*) *in vitro*. Can. J. Genet. Cytol. 5, 307-317 (1963).
2. Yu, C. K. and W. K. Sinclair. Homogeneity and stability of chromosomes of Chinese hamster cells *in vitro*. Can. J. Genet. Cytol. 6, 109-116 (1964).

MECHANISMS OF CARCINOGENESIS

Austin M. Brues, Harry Auerbach, Georgia M. DeRoche, and Linda M. Pilarski

PURPOSE AND METHODS

The purpose of this group of experiments is to investigate the carcinogenic process on the cellular level, especially in relation to cancer induction by radiation as the primary or as an adjuvant agent. While a good deal of empirical information has been gained in the past few years and while much new light has been shed on the carcinogenic process in general, most of the crucial questions about the mechanism of cancer production by radiation are no better understood now than a decade ago.⁽¹⁾ A great deal of speculation has been directed to the question of whether somatic mutation is a self-sufficient primary cause of cancer or requires other factors: this question is related to the possible effects of small amounts of radiation on large populations.⁽²⁾

The work of the present year has been carried out largely in the following categories:

1. Groups of mice were set up by Mr. Thomas Kobara in the summer of 1963 in an attempt to produce malignancy by irradiating mouse embryo skin and then transplanting the skin to adult mice by a) intramuscular inoculation into isologous mice; b) intracerebral inoculation into homologous mice; and c) cultivation in millipore diffusion chambers placed intraperitoneally. Chemical carcinogens have been reported effective using the first of these systems.⁽³⁾

2. Films have been implanted into the abdominal wall of female Sprague-Dawley rats to induce sarcomas by the "Oppenheimer technique."⁽⁴⁾ Films of various plastics and inert materials give rise to surrounding connective tissue capsules; and in a certain number of cases (reported as about 20% in most series) sarcomas develop in these capsules without further treatment. Tumors do not occur if the films are perforated or powdered before implanting. We have used films of cellophane and polyethylene, and millipore filters of 0.3-micron and of 10-micron pore size (the last is reported to produce no tumors). Tumors arise during the second year after implantation; the effect is reversible until about 6

months after implantation, i.e., removal of the films before that time prevents tumors from appearing. The areas of implantation were X-irradiated (400 R) in one experimental group 4 months and in another 8 months after implantation. The purpose of these experiments is to discover whether irradiation affects the formation of tumors from the connective tissue cells of the capsule; if tumor formation arises as a result of "spontaneous" somatic mutations in these cells, the irradiation might be expected to increase the tumor incidence by a large factor.

3. Tumors due to the development of mammary carcinomas in these rats as a result of irradiation of the abdominal wall region and tumors not associated with the implants have been removed surgically, and this material has been studied for further information about the course of development of this type of tumor.

4. About 100 female Sprague-Dawley rats have been set aside awaiting the development of spontaneous benign mammary tumors, which begin to appear in the second year of life. These rats will be irradiated locally to investigate whether cancer arises within these tumors or independently.

5. A breeding colony of hairless mice⁽⁵⁾ has been established for use in beta-ray exposures, using both external and internal sources.

6. The early stages of histologic response to protracted local irradiation have been under investigation, employing glass beads (usually of 5 or 50 microcuries of Sr^{90} - Y^{90}) embedded under the skin. This technique facilitates the study of cellular changes associated with a very wide range of dose rates and visible in a single tissue section. Laminated flat beta-ray sources are also being developed for use in similar experiments.

7. In one category of continuing experiments, the carcinogenicity of locally injected plutonium-239 is being examined comparatively in species of different size. The rationale of these experiments is to see the extent to which the incidence of induced cancer is related to the number of cells at risk, or whether the larger

organism has a lower per cell sensitivity to cancer production (as seems to be the case where bone-seeking radionuclides have been studied). It has practical importance in deriving permissible exposures to localized radiation sources in man from experiments carried out in small animals.

In preliminary experiments, the plutonium solution deposited rather early in the skeleton, and most of the resulting tumors were osteogenic. The present experiment has utilized local injections of particulate suspensions of plutonium, which are retained longer in the local area of injection. Rats and mice have been injected, and a series of female rats has been ovariectomized before injection in order to prevent the ordinarily high incidence of mammary tumors. Larger animals (rabbits and dogs) will be included in later experiments.

8. Since particulate plutonium is removed from the injection site (as inhaled plutonium particles are from the lung) mainly through incorporation into macrophages, experiments are being carried out using suspensions of plutonium-239 particles (as prepared by A. Lindenbaum for subcutaneous injection) to determine their effects on macrophages in tissue culture in collaboration with A. N. Stroud. The fate of cells which ingest these alpha-emitting particles will be studied by lapsed-time cinematography.

PROGRESS REPORT

1. No tumors have resulted from the irradiation and subsequent grafting in various ways of mouse embryo fragments.

2. Experiments with implanted plastic films have now been in progress for $1\frac{1}{2}$ years. A total of 180 rats have been implanted with plastic and millipore filters, and after 430 to 540 days, 9 sarcomas have been observed (an incidence of 5% of those originally implanted). Four of the 60 rats that were unirradiated and 5 of the 120 irradiated rats have developed tumors; thus there is no significant difference in tumor incidence at this time due to 400 R local irradiation.

The millipore filters with 10-micron apertures have shown heavy infiltration with cells, while those with 0.3-micron apertures are free of cells except where occasional cracks have occurred. Tumors have occurred in both groups. At the present time, tumors are developing at an increasing rate.

3. Benign mammary tumors have appeared in 12% of these rats, beginning at 8 months of age, and the incidence of these tumors appears unrelated to X-ray exposure. Malignant mammary tumors are consider-

ably increased in those rats irradiated over the mid-abdomen (15% as against 3%), in accordance with previous reports.

4. It appears now that particulate plutonium is a satisfactory preparation for inducing subcutaneous tumors. Results of earlier experiments, in which ionic plutonium was used, have been presented in a previous report.⁽⁶⁾ The present series of injections with particulate suspensions was started in January 1964, and local tumors are beginning to occur in rats given injections of 1.0 and 0.4 microcuries.

5. Glass beads containing Sr^{90} - Y^{90} have been embedded subcutaneously, and sections of the irradiated skin have been studied. It was shown that the resulting epidermal thickening could be accounted for entirely by enlargement of individual cells and that no increase in cell number occurred. These thickened areas were seen where the estimated absorbed dose rate was in excess of 1200-1500 rads/day; and cell enlargement continues to occur for at least two weeks after beginning irradiation. Ulceration occurs where the dose rate is about 10 times that required to produce cell enlargement.⁽⁷⁾

A similar response is seen in locally irradiated animal tumors;⁽⁸⁾ in that case the enlargement of cells can be accounted for by continued growth without cell division. In the case of the epidermis, the normal very slow growth rate of the tissue seems quite inadequate to account for the rate of cell enlargement.

Concomitant with cell enlargement, the nuclei enlarge; DNA is synthesized as well as other cell constituents; and there is a great increase in keratohyalin and apparently in keratin production.

REFERENCES

1. Brues, A. M. Radiation as a carcinogenic agent. *Radiation Res.* **3**, 273-279 (1955).
2. Brues, A. M. Somatic effects of radiation. *Proc. Am. Phil. Soc.* **107**, 1-4 (1963).
3. Rous, P. and W. E. Smith. The neoplastic potentialities of mouse embryo tissues. *J. Exptl. Med.* **81**, 597-619 (1945).
4. Oppenheimer, B. S. Malignant tumors resulting from embedding plastics in rodents. *Science* **118**, 305-306 (1953).
5. Iversen, O. and A. Evensen. Experimental skin carcinogenesis. *Acta Path. Microbiol. Scand. Suppl.* **156** (1962).
6. Brues, A. M., H. Auerbach and G. DeRoche. Effects of plutonium injected subcutaneously in the rat and mouse. Argonne National Laboratory Biological and Medical Research Division Semiannual Report, January through June 1963. ANL-6823, pp. 81-85.
7. Brues, A. M. and G. M. DeRoche. Histologic response of mouse skin to continuous irradiation. *Radiation Res.* **21**, 172 (1964).
8. Brues, A. M. and A. N. Stroud. Formation of giant nuclei in continuously irradiated tumors. *Z. Zellforsch. Mikroskop. Anat.* **64** (1), 52-62 (1964).

NEUTRON CAPTURE THERAPY OF TUMORS

Norman A. Frigerio and Lawrence Kleiman

PURPOSE AND METHODS

The theoretical possibility of applying neutron capture reactions to the treatment of neoplasms was first envisioned shortly after Chadwick's discovery of the neutron.⁽¹⁾ This idea was then extended over the years by experimental work,⁽²⁻⁵⁾ and the results obtained seemed to justify Locher's original hypothesis.

The success of such neutron capture therapy is predicated on two conditions. First, a high differential concentration of an activatable nuclide must be obtained within the neoplasm relative to the most radiosensitive surrounding tissue. Second, the nuclide must then be activated *in situ* by a flux of neutrons so that its radioactive decay products deliver a greater cytotoxic dose to the tumor than to normal tissue. The primary advantage lies in the selective destruction of the tumor. Secondly, it largely avoids the thorny questions of conventional radiotherapy of relative radiosensitivity, skin and whole body dose, excretion, handling of radioisotopes and the like. Thirdly, it provides a potential method for the destruction of metastatic tumors even without a knowledge of their precise location.

Of the nuclides originally proposed by Locher, only Li^6 , B^{10} , and U^{235} have been seriously discussed in the literature. Ionic lithium and uranium failed to localize adequately in tumors. Further, ionic uranium proved much too toxic to be considered further. Anionic boron, however, was found to localize briefly in brain tumors.⁽⁶⁾ Thus, a number of attempts to utilize polyborates, polyperhydroborates, and boronic acids in the treatment of brain tumors have been made. Unfortunately, these failed almost uniformly.^(7, 8)

These failures led us⁽⁹⁾ to re-examine thoroughly the postulates and techniques of the boron-thermal neutron systems employed in an attempt to devise more fruitful systems.

Our theoretical studies indicated that the use of epithermal neutrons (as suggested by Failla and Rossi⁽¹⁰⁾), obtained by cadmium or lithium filtration of a beam of reactor neutrons, would prove much more effective. This was demonstrated by experiment⁽¹¹⁾ and confirmed by others.^(12, 13)

Further, our studies indicated that an organouranium compound of the proper configuration would prove much more useful than B^{10} compounds. In consequence we proceeded to the synthesis of a structure indicated as useful by our theories, *viz.* U^{235} uranyl phthalocyanine tetrasulfonic acid. A therapeutic system using this compound in conjunction with a beam of epithermal neu-

trons proved successful. Complete clinical cures of mice bearing highly malignant brain tumors were effected.⁽¹⁴⁾

Unfortunately, this compound was found to possess a very long biological half-life in at least some experimental animals and to produce severe photosensitization of the subjects to even moderate light. Coincidentally, subjects were turned a deep blue-green (the color of the compound) and remained so colored for some years.

The present purposes, then, of our investigations are:

- 1) The development of alternative compounds to uranyl phthalocyanine tetrasulfonic acid free of its drawbacks.
- 2) The development of compounds which would localize in somatic tumors as well as cerebral ones.
- 3) The improvement of neutron sources, if possible, to surpass the performance of reactor epithermal beams.

PROGRESS REPORT

1) A compound, probably uranium hemiporphyrine, has been synthesized by us which appears to meet the requirements of neutron capture therapy. It is extremely stable chemically, resisting temperatures above 400° C and even such powerful reagents as boiling nitric acid. It is a moderate red in color so that, at most, patients would appear somewhat ruddy after its administration. Structurally it should be amenable to amination and subsequent coupling to serum albumin so that it could be effectively localized as described below.

2) We have found that serum albumin localizes extremely well in any site of inflammation and specifically in cerebral tumors. Further, this property is retained when organic metallic molecules are coupled to it by diazotization of aminated derivatives. This coupling has been successfully carried out with a number of aryl amines and seems to have the added effect of greatly reducing any toxicity which the parent molecule possesses. Selective concentrations fifty-fold as high in the tumor as in the blood or nearby normal tissues have been routinely obtained and maintained for periods of 16 to 48 hours. Thus, it seems highly probable that uranium hemiporphyrine-diazo-serum albumin can be synthesized and that it should prove a more useful compound than uranyl phthalocyanine tetrasulfonic acid.

3) Following our theoretical predictions, beams of monoenergetic neutrons, supplied from a Van de Graaff accelerator via the Li^7 (p, n) reaction, were directed into phantoms of tissue-equivalent gel.⁽¹⁵⁾ Conversion of part of the normal tissue content of Na^{23} to Na^{24} was then

used to follow the neutron distribution in such phantoms in a manner previously described.⁽¹¹⁾ A Rossi "W" chamber⁽¹²⁾ was simultaneously used to determine total dose to normal tissues. Curves of effective flux distribution through a phantom head are given in Figure 18 along with the curve for thermal neutrons, included for comparison.

It can be seen that beams of monoenergetic neutrons possess much more favorable penetration characteristics than thermal ones. Furthermore, the use of the very narrow beams available from the accelerator allows concentration of the neutrons into the region of interest and a rapidly diminishing total dose to the rest of the structure irradiated. This contrasts with the use of even reactor epithermal neutrons whose nature requires a

FIG. 18.—Neutron flux penetration into phantom of a human head. Curve 1, Penetration along beam axis, 100 keV neutrons; Curve 2, Penetration along axis parallel to beam and 5 cm distant, 100 keV neutrons; Curve 3, Penetration along central axis, thermal neutrons.

very broad beam for good penetration and consequent high dose throughout the irradiated structure. We have also found the gamma contamination of these monoenergetic beams to be much lower than that of either thermal or epithermal ones, i.e., less than 0.08% on a dose basis. Computations also showed that, at a U^{235} concentration in a tumor of 3.5×10^{-6} moles cm^{-3} (equal to that of B^{10} used in previous therapeutic attempts), the ratio of tumor dose to dose in normal tissues would exceed 2:1, even at the least favorable penetrations and 10:1 at penetrations between 2 and 4 cm.

4) A Monte Carlo computer program was completed which affords complete space-energy-dose histograms for all tissue volumes of therapeutic interest. These contain a model tumor whose size, position, and chemical com-

position can be varied over wide limits. The program operates successfully for a large number of neutron sources and spectra from thermal energies to 0.2 MeV. Preliminary results have borne out our original theoretical predictions⁽⁹⁾ in re the utility of neutron resonances, of gamma-emitting nuclides, and of the probable relationships of neutron energy and tumor size, location, and chemical composition. Work in this direction is continuing in an attempt to build a library of dose-energy-spectrum-beam size-penetration charts to guide future therapy.

CONCLUSIONS

Theoretical predictions originally made by us have proved valid in experiment. Systems of neutron capture therapy have been developed which utilize beams of monoenergetic neutrons in conjunction with U^{235} containing macrocyclic molecules coupled to serum proteins. They show excellent promise of surpassing the previous boron-thermal neutron-brain tumor systems by one to two orders of magnitude and of potential utility in treating somatic and metastatic tumors as well as cerebral ones.

REFERENCES

1. Locher, G. L. Therapeutic possibilities of neutrons. *Am. J. Roentgenol.* **36**, 1-13 (1936).
2. Kruger, P. G. Effects of nuclear disintegration products on neoplastic tissue. *Proc. Natl. Acad. Sci. U.S.* **26**, 181-192 (1940).
3. Kruger, P. G. Boron uptake in mouse brain neoplasm. *Radiation Res.* **3**, 1-17 (1955).
4. Zahl, P. Slow neutrons for cancer therapy. *Radiology* **37**, 673-682 (1941).
5. Farr, L. Recent advances in neutron capture therapy. *Proc. 2nd Intern. Conf. Peaceful Uses Atomic Energy*. United Nations, Geneva, 1958, p. 451.
6. Soloway, A. H. Pharmacology of boron compounds. *Progress in Boron Chemistry*. Pergamon Press, New York, 1964. Vol. 1, Ch. 4.
7. Farr, L. 1962. Private communication.
8. Sweet, W. H. 1962. Private communication.
9. Frigerio, N. A. Systems for Neutron Capture Therapy (October, 1959). Argonne National Laboratory Report ANL-6910 (1964).
10. Failla, G. and H. H. Rossi. Radiological Research Laboratory, Columbia University, Annual Report. U.S.A.E.C. Report NYO-4582 (1954).
11. Frigerio, N. A. Neutron penetration during neutron capture therapy. *Phys. Med. Biol.* **6**, 541-549 (1962).
12. Brownell, G. L. Neutron spectroscopy and dosimetry at MITR. *Neutron Dosimetry*. Intern. Atomic Energy Agency, Vienna, 1963, pp. 51-70.
13. Fairchild, R. 1964. Private communication.
14. Frigerio, N. A. Metal phthalocyanines. U.S. Patent 3,027,391 (1962).
15. Frigerio, N. A. Patterns of lethality with monoenergetic neutrons. *Biological Effects of Neutron and Proton Irradiations*. Intern. Atomic Energy Agency, Vienna, 1964. Vol. 1, pp. 117-128.
16. Rossi, H. H. Neutrons and mixed radiations. *Radiation Dosimetry*, ed. G. J. Hine and G. L. Brownell. Academic Press, New York, 1953, pp. 578-683.

ALKYL HALIDE REARRANGEMENTS

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PURPOSE AND METHODS

In the synthesis of organouranium compounds suitable for the neutron capture therapy of tumors, certain steps proposed by us proceeded via Lewis-acid-catalyzed halide rearrangements. Although the idea of a primary carbonium ion mechanism had been discredited to some extent by other workers,⁽¹⁾ it remained of importance to determine conclusively if it was, in fact, involved. If not it became important to determine what mechanisms were operative.

The purpose of these investigations, then, was to determine the mechanisms involved in the Lewis-acid-catalyzed rearrangement of simple alkyl halides.

Samples of ethyl, propyl, or butyl chlorides or bromides were incubated with varying amounts of the Lewis acids AlCl_3 , AlBr_3 , GaCl_3 , GaBr_3 , FeCl_3 and FeBr_3 as well as UO_2Cl_2 , ThCl_4 , NdCl_3 , SnCl_4 , ZnCl_2 , TiCl_4 , SbCl_3 , CeCl_3 , and $(\text{CN})_2\text{C}:\text{C}(\text{CN})_2$. Since the latter nine proved completely ineffective in promoting the rearrangement of *n*-propyl bromide, even under reflux, their use was abandoned.

The systems were maintained anaerobically at 25° C and aliquots analyzed until a steady state was reached,

and further incubation or addition of Lewis acid was without effect. Total mass-balance analysis of products was accomplished by a combination of vapor phase chromatography with conventional gasometric, volumetric and gravimetric techniques.

In addition, *n*-propyl bromide-1- C^{14} was rearranged in the presence of AlBr_3 and the products degraded specifically and scintillation counted for C^{14} by specially developed methods.⁽²⁾

PROGRESS REPORT

Ethyl Halides

Exchange between ethyl chloride and AlBr_3 occurred rapidly to yield 52.6% ethyl bromide. The converse reaction of ethyl bromide with AlCl_3 proceeded more slowly to a steady-state mixture containing only 2.1% ethyl chloride. This indicated that the alkyl-bromide bond is of greater strength than the alkyl-chloride bond.

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As has been shown⁽³⁾ such systems possess multiple pathways and produce a variety of products so that only a steady state can be obtained, dependent on the initial conditions, and not a simple Gibbsian equilibrium.

Propyl Halides

All reactions were complete within a few hours, even at low Lewis acid concentrations. The order of effectiveness in promoting halide rearrangement was found to be

That of promotion of halogen interchange between the halide and the Lewis acid, on the other hand, was

These observations (save the unexplained position of FeBr_3 for interchange) are consonant with the known effects of halogen radius, electronegativity and carbon-halogen bond thermodynamic stability.

Crossed reactions of *n*-propyl halides with Al halides gave the following distribution of products (amounts expressed in millimoles):

It was concluded from these data that a) rearrangement was the primary reaction and b) that an ionic mechanism could not be significant in this rearrangement.

Accordingly *n*-propyl bromide-1- C^{14} was rearranged in the presence of AlBr_3 to yield *i*-propyl bromide and the latter isolated and purified to constant physical properties and specific activity. The product was converted to propanol-2 with ethanolic AgNO_3 and the alcohol subjected to iodoform degradation,⁽²⁾ along with known samples of propanol-2-1,3- C^{14} . All of the C^{14} was found to lie in the 1 and 3 carbons of the product whose parent was then, of necessity, 2-bromopropane-1,3- C^{14} . This ruled out methyl migration as a mechanism and left an internal hydride-halide shift as the only reasonable mechanism.

Butyl Halides

The results of subjecting butyl chlorides to rearrangement with AlBr_3 are shown in Table 8. It is evident that

extensive methyl (and probably ethyl) migration occurs. Further, from the known order of carbonium ion stability, the probability of a carbonium ion mechanism is extremely remote. Thus, production of *n*-butyl halides from *t*, *s*, or *i*-butyl chlorides by such a mechanism would seem highly improbable.

methyl shift and so probably proceeds via an internal hydride-halide shift.

3. Rearrangement is the chief reaction in butyl systems as well. The results are not consonant with carbonium ion formation but are consonant with a high degree of methyl migration.

TABLE 8
REARRANGEMENT AND INTERCHANGE OF BUTYL CHLORIDES*

Halide	AlBr ₃	<i>n</i> -BuCl	<i>i</i> -BuCl	<i>s</i> -BuCl	<i>t</i> -BuCl	<i>i</i> -BuBr	<i>s</i> -BuBr	<i>t</i> -BuBr
<i>n</i> -BuCl.	3.70	59.4	11.4	9.4	Trace	10.0	1.5	8.3
<i>s</i> -BuCl.	4.45	Trace	22.2	48.0	25.9	Trace	Trace	3.8
<i>i</i> -BuCl.	3.78	6.8	33.0	20.4	20.4	9.0	3.6	6.8
<i>t</i> -BuCl.	4.56	12.5	5.7	14.5	36.0	10.1	11.7	9.5

* Values expressed as mole %.

CONCLUSIONS

From the data obtained we conclude:

1. Interchange is the chief reaction occurring in ethyl halide-Lewis acid-metal halide systems. This may proceed ionically.

2. Rearrangement is the chief reaction occurring in propyl systems. This cannot occur ionically or by

REFERENCES

1. Brown, H. C. Studies in stereochemistry. *J. Am. Chem. Soc.* **79**, 2302-2306 (1957).
2. Frigerio, N. A. The scintillation counting of iodoform and other alkyl halides. *Intern. J. Appl. Radiation Isotopes* **14**, 330-331 (1963).
3. Sixma, F. L. Carbonium ions in Friedel-Crafts reactions. *Rec. trav. chim.* **75**, 127-143 (1956).

DYNAMICS OF NORMAL GROWTH

Anna Kane Laird

PURPOSE AND METHODS

In our attempts to understand the dynamics of tumor growth,⁽¹⁾ we are forced to consider the dynamics of normal growth as well, since it is probable, as most workers active in the field of cancer research believe, that tumor growth will ultimately be understood as an aberration of normal growth.

The growth of normal organisms shares with that of tumors the property that it can be fitted empirically by a straight line on a log-log plot over long periods of time (i.e., that the log of body weight increases directly with the log of age).⁽²⁾ For normal growth of organisms as for tumors, the doubling times increase approximately exponentially with time. On the basis of these observations we offered the hypothesis that growth both of normal organisms and of tumors is an exponential process retarded by an exponential decay.

Our hypothesis is that growth of the normal organism is the resultant of two genetically determined exponential coefficients which operate respectively to determine 1) the magnitude of the initial exponential proliferation of the system and 2) the magnitude of the exponential decay of this primary exponential growth rate. The

interaction between these two opposing exponential processes of growth and decay of the growth rate results in the familiar properties of the growth of the metazoan organism (cf. Medawar⁽³⁾): 1) growth toward a final limiting size; 2) a limiting size that is characteristic of the species, within relatively narrow limits; 3) a specific growth rate that decreases constantly as an organism ages; 4) a decreasing rate of decrease in the specific growth rate with age. That such a mathematical relation underlies the growth of metazoans generally would account for the regular relation that is observed between the rates of growth and development to corresponding stages in different organisms.⁽⁴⁾

This model of growth is described by a Gompertz equation of the following form:

$$W = W_0 e^{(A/a)(1-e^{-at})} \quad (1)$$

in which W = size of the organism at time t , W_0 = size of the organism at time zero, and A and a are constants. This equation was developed by S. A. Tyler of this department specifically for the application of this growth model to the malignant growth of tumors and to normal growth of organisms, and it has been used extensively

during the past four years by him and by other members of this department for the analysis of the effects of radiation on the growth of the chicken and the dog.

Our method of study includes extensive surveys of the literature to collect data on the pre- and postnatal growth of organisms and of parts of organisms, for a large number of species. Such growth data are then analyzed by graphical methods and are fitted by the Gompertzian growth equation (Equation 1), using a computer program developed by M. Dipert and S. A. Tyler of this Laboratory, based on the principle of least squares fitting. Additional computer programs have been developed by A. D. Barton for algebraic manipulation of the growth equation and extrapolation of the growth curve to times of biological significance that lie outside the range of the data. Very recently, a new, much simplified method of analyzing the growth data has been developed, with A. D. Barton, which requires only the use of a slide rule and graph paper to determine two of the three independent variables of the growth equation (see below), thus making the process of growth analysis available to researchers with little or no access to a computer.

PROGRESS REPORT

Let us consider the growth of the normal guinea pig, from an early embryonic period to young adulthood. The data used for this study are those covering prenatal growth by Ibsen⁽⁶⁾ and a set of postnatal growth measurements made originally by Minot and kindly furnished by W. P. Norris of this Laboratory. Starting with the raw data, the specific growth rate can be determined by graphical means: For each pair of successive measurements, the increment in body weight (ΔW) and the increment in age (Δt) are determined by difference. The mean weight and mean time with respect to the integral of the growth curve are then approximated for each such interval by adding to the earlier weight or time a value equal to 2/3 the increment in weight or time. When the specific growth rate ($1/W \cdot \Delta W/\Delta t$) is plotted against time on a semilog graph, one obtains a straight line with a negative slope, as shown in Figure 19. If the growth of the guinea pig had followed a simple exponential course, the line in Figure 19 would be horizontal ($1/W \cdot dW/dt = k$). The negative slope observed in practice indicates however that the exponential growth process undergoes a regular decay with time that is itself exponential ($1/W \cdot dW/dt = e^{-kt}$). It is noteworthy that the data from which Figure 19 is plotted cover a range of growth of nearly 10,000-fold, from an initial value of 0.062 grams at 20 days after conception to a value of over 400 grams at about 200 days after conception. The intercept of the least squares line fitting such data as in Figure 19 gives the initial rate of ex-

ponential growth, and the slope gives the rate of exponential decay of this exponential growth as the guinea pig ages.

From the solution of the two differential equations describing the above phenomena

$$\frac{dW}{dt} = \gamma W \quad (2)$$

$$\frac{d\gamma}{dt} = -a\gamma \quad (3)$$

the growth equation (Equation 1) is immediately derived.

The mathematical properties of this growth equation have been investigated with the help of S. A. Tyler and A. D. Barton; a manuscript is currently in preparation with these coauthors, presenting the growth model and

FIG. 19.—Growth of the male guinea pig. Specific growth rate ($\Delta W/\Delta t/W$) is plotted semilogarithmically against age from conception. The line is drawn by eye; the least squares line will be computed to determine the slope (a in the growth equation, Equation 1) and the intercept (A in the growth equation), and the 95% confidence intervals. For these data, prenatal growth (upper cluster of points) and postnatal growth (lower cluster of points) are fitted approximately by the same straight line, indicating that only little if any change in the biological time of the organism occurs at birth. For many other organisms, an abrupt change in slope is observed at or near birth. A linear growth has been subtracted from the postnatal points; such linear growth is observed clearly in the young adult as the Gompertzian growth process slows to a halt, and the slope of this linear growth is easily determined at that time. For consistency's sake we assume that such linear growth is present from birth, but is obscured by the much more rapid exponential growth process that is seen early.

an extensive analysis of the mathematical properties of the growth equation. Expressions for the asymptote and the point of inflection of the growth curve have been derived, and algebraic procedures for normalizing different Gompertz curves have been established to permit direct comparison of growth processes of different organisms and their parts.

The mathematical properties of the growth equation have certain implications for the dynamics of normal growth. The successful development of procedures for the transformation of coordinates and normalization of Gompertz curves implies that the growth curves of many organisms can be superimposed, if each follows a process of exponential growth with exponential decay of the specific growth rate. Indeed, Brody,⁽⁴⁾ using a different but related growth equation, has already superimposed the latter parts of the growth curves of a great variety of organisms. Hence the growth of many species must represent examples of the same underlying biological process, programmed in terms of two exponential constants which are genetically determined for each species or breed. This model can be made more flexible to permit a regular change to another set of parameters at some predetermined stage of development, as, for example, at birth (preliminary analysis indicates that for the chicken and the mouse, and for another breed of guinea pig, such reprogramming does occur, at or near the time of birth).

Let us consider another of the mathematical properties of the growth equation (Equation 1), namely that α is the constant factor by which time must be multiplied in order to normalize the time coordinates of different growth curves. This fact implies that when the growth of two organisms can be described by a Gompertz equation of the form used here, the time scales of the two organisms are related as the values of α in the specific equation for each organism. That is, α may be considered to be the coefficient of biological time. Metaphorically, it is as though the growth of the organism were timed by a clock, which is wound up at or soon after conception, and the clock then runs down at a characteristic constant exponential rate ($=e^{-\alpha t}$), rather than at a linear rate, as real clocks do. In this relation, α subserves the same function as the exponential decay constant, k , in Brody's analysis of the growth of normal organisms beyond the inflection point⁽⁴⁾ but α is applicable to growth before the inflection point as well.

On the basis of our model we can make predictions of experimental findings: The constants of the growth equation should be alterable independently of one another. If W_0 is the effective reproductive mass at any stage of growth, then we might anticipate that radiation and certain other toxic agents, notably carcinogens,

would reduce this mass, in accordance with the known effects of these agents on the reproductive units (proliferating cells) of such systems as bacterial and protozoal cultures. After a critical period of cell death, and when the acute toxic effects of such agents have worn off, we would expect the organism to grow much as it had been growing before, but from a depressed baseline, such growth resulting in a depression of the asymptote, and a reduction in the size of the adult organism. Preliminary analysis of experiments in this Laboratory on the effects of certain toxic agents on the growth of animals, has confirmed this prediction (effects of the liver carcinogen, 2-acetylaminofluorene, on the growth of rats; effects of whole-body radiation on the growth of chickens). In other cases we might anticipate that α might be altered independently; if α is the coefficient of biological time, and if growth may be thought of as being timed by a clock running down at an exponential rate ($=e^{-\alpha t}$), then the rate at which the clock slows could be changed sharply at any time, or the whole process might even be stopped temporarily. Such a resetting of the clock is seen naturally at or near birth in the chicken and mouse (Laird, unpublished findings) and has been produced experimentally by severe dietary restriction.^(6, 7) That the clock may be turned on again after a period of little or no growth, with no essential change in its rate of running down, is indicated by the experiments of Osborne and Mendel,^(6, 7) in which, after prolonged dietary restriction, animals returned to a normal diet grow to normal adult size according to an approximately normal growth curve.

If our model of growth is correct in detail, that is, the major growth of the organism is due to exponential proliferation of stem cells, retarded by an exponential decay in the rate of this growth, then parts of the organism should show similar patterns of growth, and the growth of different parts of an organism should follow a regular relation. Huxley's Law of Relative Growth⁽⁸⁾ (log size of part A plotted against log size of part B generally yields a straight line) is fully explained on the basis of our model, since two Gompertz equations will yield a straight line on a log-log plot, if the value of α is the same for both.⁽⁹⁾ If α is the coefficient of biological time for the organism as a whole, one would expect that in most cases the value of α would be the same for different parts of the organism. Preliminary analysis by the graphical method (above) indicates that for the chick embryo, the main body parts (head, trunk, fore- and hind-limbs) grow in such a way that the specific growth rate plotted against time yields a series of parallel lines on a semilog plot; i.e., the specific growth rates of these parts decay at very nearly the same semilog rate (they have the same value for α in Equation 1), but the values for the intercepts (A in Equation 1) are different.

CONCLUSIONS

No exceptions have been found so far to the application of our growth equation to the growth of whole organisms and their parts, both pre- and postnatal.

Since different Gompertz curves can be superimposed by a simple algebraic manipulation of the parameters, the fitting of so many growth processes by our equation implies that these are essentially identical biological processes, differing only in the time and size scales on which they are carried out.

Preliminary explorations have indicated already that the parameters of growth may be affected individually by experimental agents, including carcinogens and radiation, and hence that this growth equation provides a very useful tool in radiobiology and cancer research.

DYNAMICS OF TUMOR GROWTH

Anna Kane Laird

PURPOSE AND METHODS

It is generally accepted that tumors represent a deviation from the normal growth of the organism, yet little is known of the laws that govern either normal growth or the growth of tumors. The purpose of the present study is to identify the appropriate growth laws of the normal organism and to define the specific aberration(s) of these laws that tumor growth represents. It is hoped that such an understanding will direct our search for a practical means of controlling tumor growth.

We have approached this problem from two directions. In previous work⁽¹⁻³⁾ we studied the cell proliferation occurring in preneoplastic livers and established that the net accumulation of cells is mathematically a linear process, rather than the exponential process one might expect, and that the rate of this linear proliferation is a function of the dose of the carcinogen. Such preneoplastic cell proliferation ceases soon after exposure to the carcinogen is stopped.⁽³⁾ Since the time of appearance of tumors can be delayed by such withdrawal of the carcinogen, we believe that this proliferation is an important factor in the eventual production of tumors.

More recently we have turned to the literature for data that can be used for a more extensive mathematical analysis of malignant growth. Our method of study includes extensive search of the literature to collect measurements of a wide variety of tumors, made by many different investigators, in the hope of finding the com-

REFERENCES

1. Laird, A. K. Dynamics of tumor growth. *Brit. J. Cancer*, **18** (3), 490-502 (1964).
2. Osgood, E. E. Development and growth of hematopoietic tissues: with a clinically practical method of growth analysis. *Pediatrics* **15**, 733-751 (1955).
3. Medawar, P. B. Size, shape and age. *Essays of Growth and Form*, ed. W. E. LeGros Clark and P. B. Medawar. Oxford University Press, Oxford, 1945, pp. 157-187.
4. Brody, S. *Bioenergetics and Growth*. New York, Reinhold Publ. Corp. 1945.
5. Ibsen, H. L. Prenatal growth in guinea pigs with specific reference to environmental factors affecting weight at birth. *J. Exptl. Zool.* **51**, 51-91 (1928).
6. Osborne, T. B. and L. B. Mendel. The suppression of growth and the capacity to grow. *J. Biol. Chem.* **18**, 95-103 (1914).
7. Osborne, T. B. and L. B. Mendel. The resumption of growth after continued failure to grow. *J. Biol. Chem.* **23**, 439-54 (1915).
8. Huxley, J. S. *Problems of Relative Growth*. The Dial Press, New York, 1932.
9. Lumer, H. The consequences of sigmoid growth for relative growth functions. *Growth* **1**, 140-154 (1937).

mon nature of all tumor growth. Such growth data have been analyzed by graphical methods, and have been fitted by our growth equation (see below) using a computer program developed by M. Dipert and S. A. Tyler of this Laboratory. Additional computer programs have been developed by A. D. Barton for algebraic manipulation of the growth equation and extrapolation of the growth curve to times of biological significance which lie outside the range of the data.

PROGRESS REPORT

Several years ago we observed that the growth of representative tumors of the mouse, rat, and rabbit can be fitted empirically by a straight line on a log-log plot (i.e., that the log of tumor size increases directly with the log of time of tumor growth). On the basis of this observation, and of the further observation that the doubling times of such tumors increase exponentially during growth, we developed a model according to which tumors grow by an exponential proliferation of tumor cells retarded by an exponential decay. This model is described exactly by a Gompertz equation of the following form:

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

in which W = tumor size at time t , W_0 = tumor size at time zero, and A and α are constants. This equation was developed for the purposes of this study by S. A. Tyler of this Laboratory, and this growth model and its equation have been used extensively by him and other

members of this department for the analysis of the effects of radiation on the growth of the chicken and the dog.

Using this equation to study the growth of tumors, we have found that the growth of 19 tumors of the mouse, rat, and rabbit, including both primary and transplanted tumors, can be well described by this equation,⁽⁴⁾ and hence, that our model appears to describe a common property of tumors. More recently we have developed an algebraic procedure for "normalizing" the growth data of these tumors; Figure 20 shows the data for all 19 tumors of this sample converted to a single plot by appropriate adjustment of the size and time scales, using

back of the inflection point. Since the amount of retardation characteristic of the Gompertz function increases as growth proceeds toward the asymptote, the rat tumors in general experience much less retardation than do the mouse tumors; conversely, it is because the rat tumors show much less retardation than do the mouse tumors that they fit the Gompertz curve so far to the left. The fitting of the curve is essentially a fitting of contours.

It is fortunate that our sample is so extensive, because it establishes that some tumors show strong retardation within the life of the host, while others, although conforming to the Gompertzian model, deviate

FIG. 20.—A normalized Gompertzian plot, in which the growth data for 19 examples of 12 different tumors of the rat, mouse, and rabbit have been superimposed, after adjustment of the units, on the time and size axes; the point of reference, at the intersection of the two scales, is the inflection point of the growth curve. The ranges of growth of three individual tumors (El₄, 6C₃ HED, and W26b1) are indicated by vertical arrows.

the inflection point of the theoretical curve as the point of reference. The degree of retardation (exponential decay of the rate of growth) observed in this sample of tumors considered as a whole extends from what is statistically indistinguishable from zero in the case of one tumor to retardation so great that the last data points show no consistent increase in size of the tumor. However, although the growth of all these tumors can be approximated by a Gompertz curve, the part of such a curve followed by a given tumor before the death of the host is limited, and to some extent is characteristic of the tumor. In general the mouse tumors occupy a region near the inflection point and extend well toward the asymptote, while many of the rat tumors occupy a region well

relatively little from exponential growth during the period of observation. This finding lends further support to the conclusion expressed earlier⁽⁴⁾ that the Gompertzian retardation of tumor growth is not a fortuitous result of the failure of the dying host to afford nutritional support to the tumor, but is a characteristic property of the growth of tumors in the animal host.

By algebraic analysis of the growth equation, it was determined that the rates of growth of different tumors can be compared if the comparison is made at corresponding points in the growth process. The relative position of any point on the growth curve can be defined by the ratio of the tumor size at that point to the computed tumor size at the asymptote, and points on differ-

ent Gompertz curves are corresponding points if the ratio of the tumor size at each of these points to the asymptote of its own curve is the same. When the growth rates of the 19 tumors of this sample were compared, it was found that at corresponding points in the growth process, the rat and rabbit tumors have generally passed through many more cell generations than have the mouse tumors, and hence are larger. The time required to double the tumor size is a function of the instantaneous rate of production of new cells in relation to the number of cells already present, and hence, varies from tumor to tumor, but is generally greater for the rat tumors than for the mouse and rabbit tumors.

When the growth curve is extrapolated back to a tumor size of one cell, we find plausible values for the time at which each tumor would have had this size. In particular, the computed time at which the primary tumors of this sample would have started growing as a single cell is greater than 23 days after the start of exposure to the carcinogen, a time that agrees well with the findings for other carcinogenic processes.

The number of doublings of tumor size required for a tumor to grow from a single cell to the computed upper limit of growth is essentially constant at one value for all but one of mouse tumors, and at another value for all examples of one of the rat tumors. A similar constancy is found for the time required for such growth when the

time scale is normalized. Hence there appears to be a strong tendency for different examples of the same tumor to be characterized by a predetermined range of growth from one cell toward a final limiting size, which can be readily computed, but which is not usually reached before the death of the host.

CONCLUSIONS

These relations suggest strongly that a tumor implanted into a new host grows as though it were a community of cells derived in that host from a single cell at some definite time before implantation. It grows as though it were a single organism, rather than as a population of dissociated individual cells, as presumably bacteria and other free cells do when inoculated into a new culture medium. This relation suggests further that the host plus tumor represents a new, integrated system of growth whose nature we do not as yet understand.

REFERENCES

1. Laird, A. K. and A. D. Barton. Cell growth and the development of tumours. *Nature* **183**, 1655-1657 (1959).
2. Laird, A. K. and A. D. Barton. Cell proliferation in precancerous liver: linear growth curve. *Nature* **188**, 417-418 (1960).
3. Laird, A. K. and A. D. Barton. Cell proliferation in precancerous liver: relation to presence and dose of carcinogen. *J. Natl. Cancer Inst.* **27**, 827-839 (1961).
4. Laird, A. K. Dynamics of tumor growth. *Brit. J. Cancer*, **18** (3), 490-502 (1964).

AN APPROACH TO THE UNDERSTANDING OF THE CARCINOGENIC PROCESS

Harold Sutton

PURPOSE AND METHODS

This investigation is directed at testing in a suitable *in vivo* carcinogen target tissue system the hypothesis that the carcinogenic response in a tissue, expressed for example by tumor incidence, will vary with the level of DNA synthesis at the time the target tissue is exposed to the carcinogen.

There is some evidence relevant to this hypothesis. DNA synthesis in the skin has been found to have a diurnal variation. When the skin of male Swiss mice is painted with the chemical carcinogen, 7,12-dimethylbenz(a)anthracene (DMBA), more papillomas per animal are induced when the carcinogen is applied at the time of greater DNA synthesis in the skin. However, the percentage of individual mice developing neoplasms is the same whether the carcinogen is applied during high or low DNA synthesis levels.⁽¹⁾

We have considered the following factors to be important in the design of an experiment to explore this hypothesis:

1. Groups of individuals in the species of animals selected should have a consistent average target tissue response to a fixed dose of the carcinogen.
2. The dose level of the carcinogen should produce a consistent range of response in individual animals within the group. For example, some animals should have no tumors and other animals should have single or multiple tumors in the target tissue.
3. The DNA synthetic activity in the target tissue should fluctuate physiologically between distinct and consistent levels.
4. A reliable assay of DNA synthesis is needed.
5. Ultimately, the level of DNA synthesis in the target tissue of the individual animal should be predictable on some basis, without disturbing the physiology of the animal. As nearly as possible, environmental factors which might change DNA synthesis should be controlled.
6. The tumors should appear within a consistent period of time following exposure to the carcinogen.
7. The environment during the latent period should be similarly controlled for all animals.
8. The incidence of tumor in the target tissue should be low or absent in the untreated control animals.

For our experiments we have chosen X-irradiation and rat breast epithelium as the *in vivo* carcinogen

tissue system. A single exposure of 400R, 250 kV, whole-body X-ray is administered to young mature Sprague Dawley female rats. There is an 80% incidence of mammary tumors 11 months after treatment. Some rats had multiple tumors. Castrate rats are tumor free and untreated rats have a very low incidence of neoplasm.⁽²⁾ This system offers the advantage of administering the carcinogen in a short time interval when the tissue of interest could be at distinctly different levels of DNA synthesis. By contrast, in a system involving a chemical carcinogen, the exposure to the carcinogen is less easily defined since there may be factors of slow or uncontrolled absorption or excretion. In testing the hypothesis, it is awkward to interpret data from a system in which prolonged exposure to the carcinogen may be accompanied by drastic changes in the level of DNA synthetic activity.

PROGRESS REPORT

Currently, we are studying the changes in quantity of DNA synthesis in rat breast. Bullough⁽³⁾ and Lagachev⁽⁴⁾ related changes in breast mitotic counts with changes in the vaginal smear. They concluded that there were patterns of breast epithelial proliferation which could be related to the ovarian cycle. Taylor⁽⁵⁾ used the uptake of the DNA precursor, tritiated thymidine, as a measure of DNA synthesis. Our initial problem is to relate breast DNA synthesis, as assayed by the uptake of tritiated thymidine, with cyclic ovarian hormonal changes as reflected in the vaginal smear.

The unstained vaginal smears of 50-day-old Charles River (Sprague-Dawley derived) rats were examined daily for a 2-week period. A few drops of saline from a pipette were refluxed in the vagina and withdrawn. The suspended cellular debris was examined under a low power light microscope and, depending upon cytological criteria, the rat hormonal status was classified in one of the four cycle phases: proestrus, estrus, metestrus, and diestrus. The vast majority of individual rats had regular 4- to 5-day cycles over a 2-week period. At ages 61 to 65 days, one microcurie per gram body weight of 0.36 Ci/mM of tritiated thymidine in saline was administered via tail vein in the unanesthetized rat. All injections were carried out between the hours of 10:00 A.M. and 3:00 P.M. The smears were checked 3 hours prior to injection and the final classification and assignment of the rat to a hormonal cycle phase was made by re-smearing just prior to the injection of the tritiated thymidine. The animals were chosen to give equal representation in all four phases of the cycle. Two hours later the rats were sacrificed by cervical spinal dislocation. The three abdominal-inguinal pairs of mammary fat pads which include the mammary duct and alveolar system were dissected from each rat, then frozen and stored until extraction by the procedure used by Tucker

and Reece.⁽⁶⁾ The mammary glands on removal from storage were thawed and extracted for 24 hours in 95% ethanol and 24 hours in anhydrous ether, dried to a constant weight, and ground to a powder in a Wiley mill.

Twenty-milligram aliquots of this material were combusted in duplicate by the modified Schöniger combustion technique.⁽⁷⁾ The resulting tritiated water was mixed with a solvent-fluor system (toluene, alcohol, Liquifluor) and counted in a Packard Tricarb liquid scintillation spectrometer. The counting efficiency of this system was about 10%. The lowest samples yielded counts which were eight times those of background. Replicate combustions were in good agreement.

Despite some individual variation in tritiated thymidine uptake by animals in any one cyclic hormonal phase, average values indicate that there is approximately a two-fold increase in DNA synthesis in metestrus as compared with diestrus. Proestrus and estrus are at an intermediate level. Material from these 85 animals will be studied autoradiographically and a chemical isolation of DNA followed by scintillation counting will be carried out to further clarify this pattern.

It would appear that the relationship of the ovarian cycle to DNA synthesis in the breast can be effectively studied by combining the vaginal smear technique with the combustion of tritiated-thymidine-labeled breast tissue. The peak of DNA synthetic activity occurs during early metestrus and the lowest activity is in diestrus. Such information seems essential to testing the hypothesis that tumor induction will vary with the level of DNA synthetic activity in target tissue during exposure to the carcinogen. Other characteristics of the rat epithelium, X-irradiation model also seem favorable for testing this hypothesis.

REFERENCES

1. Frie, J. V. and A. C. Ritchie. Diurnal variation in the susceptibility of mouse epidermis to carcinogen and its relationship to DNA synthesis. *J. Natl. Cancer Inst.* **32**, 1213-1220 (1964).
2. Bond, V. P., C. J. Cronkite, S. W. Shellabarger, J. Furth and R. A. Conrad. Mechanism of induction of mammary neoplasms in rats by radiation: Relation to dose and ovarian status. *Progress in Nuclear Energy, Series VI*, Vol. 2. Pergamon Press, London, 1959. pp. 131-145.
3. Bullough, W. S. Mitotic activity in the adult female mouse, *Mus musculus*^L. A study of its relations to the oestrous cycle in normal and abnormal conditions. *Phil. Trans. Roy. Soc., London.* **231**, 453-517 (1946).
4. Lagachev, S. S. Proliferation of the epithelium of mammary glands in various stages of the sexual cycle. *Bull. Exptl. Biol. Med.* **46**, 1132-1136 (1958).
5. Taylor, J. H., P. S. Woods and W. L. Hughes. The organization and suppletion of chromosomes as revealed by autoradiographic studies using tritium-labeled thymidine. *Proc. Natl. Acad. Sci.* **43**, 122-127 (1957).
6. Tucker, J. H. and R. P. Reece. Nucleic acid estimates of mammary tissue and nuclei. *Proc. Soc. Exptl. Biol. Med.* **111**, 639-642 (1962).
7. Schöniger, W. Eine mikroanalytische Schnellbestimmung von Halogen in organischen Substanzen. *Mikrochim. Acta* **1**, 123-129 (1955).

MOLECULAR AND ULTRASTRUCTURAL ASPECTS OF PLASTID DEVELOPMENT AND CHLOROPHYLL PRODUCTION IN STAGE-CHARACTERIZED POPULATIONS OF THE CHLORELLA MUTANT C-10

Gary W. Bryan and Charles F. Ehret

PURPOSE AND METHODS

Our purpose is to characterize the ultrastructural changes which accompany chlorophyll production in cells initially lacking both chlorophyll and lamellar structures.

To achieve this end, precise determination of the stage of cell and plastid development must be obtainable. The cultures are grown in darkness at 28°–29° C in a flask containing liquid media. Aliquots are counted and size relationships of the population are measured to indicate the maturity of the cell population. Acetone extractions and optical density readings are used for pigment determinations.⁽¹⁾ Ultrastructural changes are determined by fixation with 2% KMnO₄ or 1% OsO₄, embedding in Vestopal, sectioning, and viewing under the electron microscope. Autoradiographic techniques are employed to determine amino acid incorporation during plastid development.

PROGRESS REPORT

In order to determine whether changes in the distribution of cell sizes occur during the growth of the population the Coulter counter was employed. If changes were observed, could they be correlated with the stage of cultural maturity? With these questions in mind we made size determinations while counting the population.

Figure 21 shows exponential growth between 18 and 66 hours for dark-grown C-10; Figure 22 shows a strong correlation between cell size distribution and cultural maturity. During the 40 hours of exponential growth over half the cells are very large, being $\geq 100 \mu^3$. However on a cell-stage basis, the population is quite heterogeneous, since many of the larger cells already contain 2, 4, 8, or 16 fissionots (autospores) ready to be released.⁽²⁾ By the 72nd hour the autospores are released, and further divisions cease, giving a more uniform population especially suitable for induction experiments. Stage-characterized populations such as this one, with 70% of

cells in the $10\text{--}50 \mu^3$ range were used in the following experiments.

To learn whether pigment production and plastid development can be inhibited by blocking the formation of messenger RNA and/or protein synthesis an experiment was run using actinomycin D, puromycin,⁽³⁾ and chloramphenicol⁽⁴⁾ as the antimetabolites of choice. If protein production were inhibited by one means or another, then a reduction of chlorophyll would be expected, and some changes at an ultrastructural level would be observed in the plastid.

FIG. 21.—Curves showing the relationship of cell number to age of culture for *Chlorella vulgaris* mutant C-10 grown in darkness on defined media containing 0.2% glucose and 0.1 ppm iron at 28° C.

Cells were suspended in varying concentrations of the antimetabolites, and aliquots were taken for chlorophyll and ultrastructural determinations. Figure 23 shows the concentration of chlorophyll per cell in the various treatments relative to that found in the untreated cell. These and similar data show that chloramphenicol and actinomycin D cause a 40% reduction of chlorophyll production while puromycin only reduces chlorophyll

FIG. 22.—Curves showing the cell size distribution relative to age of culture in dark grown C-10 cells.

production by 20%. Furthermore, plastid development does not proceed to the degree which untreated cells do when exposed to the same source of light for a comparable duration. In addition to the quantitative difference induced by each antimetabolite, actinomycin D causes a striking qualitative difference in the ultrastructural configurations of the proplastid regions of the cell in the form of clusters of vesicles which appear similar to the dispersed crystalline bodies of light-induced higher plants.⁽⁵⁾

If one assumes size is related to stage⁽²⁾ and stage to age of cell (with age referential to some particular transcription point in a transcription-translation process essential for plastid protein synthesis), then it is not unreasonable to expect that the sensitivity of the cell to the antimetabolites (actinomycin D, puromycin, and chloramphenicol) should show a direct correlation with size. For example, one may assume that within a hetero-

geneous population, cells of a certain size range have just synthesized the particular m-RNA's required for protein synthesis in plastid development, whereas cells of all other size ranges have nearly or entirely depleted their supplies. For actinomycin D, the uninhibited cells (asymptote Figure 23) may represent the former category, and the "have-not" cells, lacking m-RNA and readily inhibited from synthesizing it by actinomycin D, may be members of the reciprocal population. From this, it appears reasonable to deduce that the sensitive 40% fraction of the population is comprised of at least that 30% of the population larger than 50 μ^3 (Figure 22). The lesser inhibition by puromycin may in like manner be composed of only that 20% of the population comprised of cells larger than 100 μ^3 . In order to prove such stage-age dependent syntheses, additional experiments will be attempted with the appropriately selected cell categories. Another alternative to bear in mind is that the blockage is only partial and possibly independent of cell age.

FIG. 23.—Dose response curves showing the percentage of chlorophyll production in C-10 cells after a 16-hour illumination with red fluorescent light relative to the concentration of puromycin, actinomycin D, or chloramphenicol.

In order to show at the ultrastructural level that protein synthesis occurs in the plastid during the greening process, C-10 cells were suspended in tritiated leucine (1.0 millicuries/ml) for a portion of the illumination period.⁽⁶⁾

In Figure 24 the heavy dark line shows the duration of the pulse and the lighter line the duration of the post-pulse period. Pulses of $\frac{1}{2}$, 2, or 6 hours followed by a 4-hour postpulse period were given to cells which had received 0, 2, or 4 hours of GE red light.⁽⁷⁾ The position

of the line is indicative of the number of silver grains per cell section.⁽⁸⁾ One sees that the duration of the pulse has less influence than the stage at which it is given. When the pulse is given after 3 to 6 hours of illumination (Figure 25), the period of maximum chlorophyll production,

FIG. 24.—The average number of silver grains per cell section as determined on electron microscopic autoradiographs of dark-grown C-10 cells which were suspended in tritiated leucine (1.0 millicurie/ml) during their pulse interval. The cells were given 0, 2, or 4 hour prepulse illumination which was followed by a pulse of $\frac{1}{2}$, 2, or 6 hours. The heavier line shows the pulse time and the lighter line the postpulse time after which the cells were fixed.

little difference is observed between the different pulse durations.

Neither the duration or the stage of development seems to have any influence on the distribution of the tritiated leucine in the cell (Figure 26) with $\sim 60\%$ in

FIG. 25.—Chlorophyll concentration as measured by the optical density at 663 $m\mu$ of acetone extracts, relative to time after initiation of chlorophyll synthesis is shown by the line. The rate at which chlorophyll was produced (shaded area) reaches a maximum before the 4th hour following induction.

FIG. 26.—The distribution of silver grains within the cell relative to the duration of the pulse in tritiated leucine (1.0 millicurie/ml) as determined on electron microscopic autoradiographs. (Δ = 0 hours, $\square$ = 2 hours, and $\circ$ = 4 hours prepulse illumination.)

the chloroplast, ~30% in the cytoplasm, ~10% in the nucleus, and a few grains in the vacuole. However, the uniform distribution of silver grains does not rule out the possibility that some of the cytoplasmic and nuclear protein into which the tritiated leucine is incorporated is transient rather than structural protein. Although there are more silver grains over the plastid than over the remainder of the cell, the intracellular distribution relationships remain constant indicating that induction has influenced not merely the molecular economy of the proplastid alone, but that reactions in every major cell compartment have changed. Had plastid protein synthesis been most rapid during or prior to the period of accelerated chlorophyll production more tritiated leucine should have been incorporated in the plastid.

CONCLUSIONS

In developmental studies in order to meet the required knowledge of cell age, two methods are generally employed: the single cell method (the one of choice)⁽⁹⁾ or the synchronized population method.⁽²⁾ In the present study we have shown the effectiveness (and suggested some of the pitfalls) of a third method, that of the stage-characterized cell population. If one may assume, as we have done, that size and age are directly correlated, then one should be able to draw valid conclusions regarding age-stage dependent sensitivities to antimetabolites from comparisons between the dose response curves for the respective antimetabolites and the size distribution spectrum for a given population. Having done this, we tentatively concluded that protein synthesis and m-RNA are essential prerequisites of photo-induced chlorophyll synthesis, and that in a normal population 60% of the cells have passed the point of transcription. Consistent with this view, suggesting an involvement not merely of the proplastid but of the entire cellular economy in the induction process, are the

results with electron microscopic autoradiography. They show, following induction, incorporation of tritiated leucine into nuclear and cytoplasmic as well as into proplastid and plastid structures, each dependent upon stage.

Studies in progress are designed to distinguish between the roles of structural and transient proteins in this process.

We wish to acknowledge the skilled assistance of Judy Alblinger.

REFERENCES

1. Bryan, G. W. and L. Bogorad. Protochlorophyllide and chlorophyll formation in response to iron nutrition in a *Chlorella* mutant. *Studies on Microalgae and Photosynthetic Bacteria*, ed. Japanese Society of Plant Physiologists. The University of Tokyo Press, Tokyo (1963).
2. Tamiya, H. Growth and cell division of *Chlorella*. *Synchrony in Cell Division and Growth*, ed. E. Zeuthen. Interscience Publishers, New York (1964).
3. Granick, S. Induction of the synthesis of δ -aminolevulinic acid synthetase in liver parenchyma cells in culture by chemicals that induce acute porphyria. *J. Biol. Chem.* **238**, PC2247-PC2248 (1963).
4. Smillie, R. M., W. R. Evans and H. Lyman. Metabolic events during the formation of a photosynthetic from a nonphotosynthetic cell. *Brookhaven Symp. Biol.* **16**, 89-108 (1963).
5. Klein, S., G. Bryan and L. Bogorad. Early stages in the development of plastid fine structure in red and far-red light. *J. Cell Biol.* **22**, 433-442 (1964).
6. Bryan, G. W. and J. A. Alblinger. Molecular and ultrastructural aspects of plastid development and chlorophyll production in the *Chlorella* mutant C-10. Argonne National Laboratory Biological and Medical Research Division Semiannual Report, January through June, 1963. ANL-6823, pp. 161-164.
7. Bryan, G. W. Protochlorophyllide *a* metabolism and chlorophyllide *a* production by a *Chlorella* mutant in response to iron and spectral exposures. Doctoral thesis. The University of Chicago, 1962.
8. Ehret, C. F., N. Savage and J. A. Alblinger. Patterns of segregation of structural elements during cell division. *Z. Zellforsch. Mikroskop. Anat.* **64**(1), 129-139 (1964).
9. de Haller, G., C. F. Ehret and R. Naef. Technique d'inclusion et d'ultramicrotomie, destinée à l'étude du développement des organelles dans une cellule isolée. *Experientia* **17**, 524-526 (1961).

FINE STRUCTURE OF CENTRIFUGED AMOEBA NUCLEI

Edward W. Daniels and Evelyn P. Breyer

PURPOSE AND METHODS

The purpose of this project is to learn more about the basic nature of interphase cell nuclei by stratifying the nucleoplasm. Information about the quantity and quality of subnuclear components (RNA, DNA, protein, etc.) is sought by the method of centrifuging at high gravitational forces. Relatively little work has been done on this subject.^(1, 2)

The uninucleate *Amoeba proteus* and the multinucleate *Pelomyxa illinoisensis* were used in this study. Various types of dense media for cushioning the cells were used in the bottom of the centrifuge tubes. The best stratification was achieved at 140,000 g and 15° C for 4 hours, with a cushioning medium of nontoxic, dense, silicone oil. The amoebae were prepared for electron microscopy and examined as previously described.⁽³⁾

PROGRESS REPORT

A great improvement over our earlier work with glycerol⁽⁴⁾ was achieved with oil. The nucleoplasm and nuclear membrane appeared as well preserved as that of the normal controls. The nucleoplasm in the two species of amoeba separated into similar but not identical strata. In *A. proteus* the nucleolar material concentrates in two strata in the centrifugal portion of the nucleus. Both strata are quite homogeneous and are extremely electron opaque. The upper stratum is more opaque than the lower and is about one third the volume of the lower. The nucleoplasm above this nucleolar material does not separate into very distinct strata. Helices and aggregations of large granules are found in the region above and adjacent to the nucleolar area; the finest granules occupy the most centripetal portion of the nucleus.

The nucleus of *P. illinoisensis* usually contains three distinct strata. Nucleolar material occupies the centrifugal portion of the nucleus in one homogeneous and very electron opaque stratum. The middle stratum is comprised of large granules with interspersed helices toward the centrifugal region or nucleolar material. The centripetal stratum consists of fine granules which are

relatively uniform in size and dispersion. A small fourth stratum is sometimes seen. It consists of granules intermediary in size between those in the large and the fine granular strata and occupies an area between the two.

CONCLUSIONS

A number of subnuclear components have been separated by high centrifugal force into distinct strata and studied with the electron microscope. *A. proteus* contains four strata two of which appear to be nucleolar in nature. *P. illinoisensis* contains three strata (and sometimes four); the centrifugal stratum appears to be nucleolar.

REFERENCES

1. Bouck, G. B. Stratification and subsequent behavior of plant cell organelles. *J. Cell Biol.* **18**, 441-457 (1963).
2. Katzberg, A. A. and G. T. Daye. Survival of the isolated embryonic heart when exposed to high relative centrifugal forces. School of Aviation Medicine, Brooks Air Force Base, Texas 6-19, 1-9 (1960).
3. Daniels, E. W., E. P. Breyer and C. W. Kischer. Intranuclear stratification in the amoeba, *Pelomyxa illinoisensis*. Argonne National Laboratory Biological and Medical Research Division, Semiannual Report, January through June, 1963. ANL-6823, p. 169.
4. Daniels, E. W., E. P. Breyer and C. W. Kischer. Fine structure of centrifuged amoeba nuclei. *J. Protozool.* **11**, 13-14 (1964).

DEVELOPMENTAL AND ULTRASTRUCTURAL STUDIES OF CELL ORGANELLES

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PURPOSE AND METHODS

Recent electron microscopic studies of single cells of known age have established that the principal organelles and organelle systems of the cell surface in *Paramecium* arise by an orderly developmental process from more elementary structures, without the intervention by division or bipartition of the ancestral organelles.⁽¹⁾ Our purpose is to continue along the lines already specified⁽¹⁻³⁾ toward an understanding of the mechanisms controlling growth and form, especially at the organellar and cellular levels. We place particular emphasis on measures of the following properties: 1) molecular stability and composition of organelles, 2) generation time of organelles arising from preorganelles, and 3) patterns of distribution of new organelles within preexisting organelle complexes and systems. Consequential to the data in these categories, we conclude with a theoretical treatment of the role of one-dimensional supertemplates and cytotaxis⁽⁴⁾ in specifying the three dimensional forms of cells.

PROGRESS REPORT

Molecular Stability and Composition of Organelles

When *Paramecium* divides, the proter (anterior fission) retains the old gullet, and the opisthe gets the newly synthesized one.⁽¹⁾ In a lineage study⁽⁵⁾ we may assume that a cell grown during f_0 in the presence of a labeled amino acid will have the newly synthesized structural proteins of its progeny at f_1 labeled, whereas ancestral structural proteins will remain unlabeled. Thus, the structural proteins of the newly synthesized cilia of the opisthe's gullet should be labeled, whereas the structural proteins of the proter's ancestral gullet ciliature should remain unlabeled. This expectation further presupposes (not unrealistically, Reference 5) the retention without significant loss, replacement, or growth of ancestral cilia.

Cells of known age (10 minutes) were grown in medium containing 750 $\mu\text{C}/\text{ml}$ tritiated leucine⁽⁵⁾ for a single generation interval (12 hours). When these divided, their proters and opisthes were identified and transferred into medium containing unlabeled leucine as

chaser, where they remained for 2 hours. Each cell was then fixed, embedded and sectioned by the single-cell technique.⁽⁶⁾ Figures 27 and 28 are electron microscopic autoradiographs of the gullet regions of a proter and an opisthe respectively after exposure times of 3 days (Ilford L-4, Reference 5). It is clear that in these f_1 cells, two hours after a twelve-hour pulse given to their f_0 progenitor, the gullet cilia of each fission are labeled. However, the axonemes and the kinetosomes of the opisthe are about twice as heavily labeled as those of the proter. Table 9 summarizes the data from two lineages

of similar material in terms of grain counts associated with either axonemes or kinetosomes. Nearly 10,000 axoneme cross sections were counted on the 100 cell sections tabulated here. From this result we conclude that about half of the newly synthesized protein of the cilia-ture of the opisthe's gullet and all of that labeled protein present on proter's gullet cilia at two hours after the long pulse is of a transient, nonstructural, and relatively short-lived and unbound nature. Kinetic studies now in progress will establish the decay constants for the transient protein(s), and may enable a further distinc-

FIG. 27.—Electron microscopic autoradiograph illustrating a > 12 hour old gullet of a proter cell of *Paramecium*. Silver grains overlying old axonemes and kinetosomes of proter's gullet originate from tritium-labeled leucine which is incorporated in transient or nonstructural protein.

TABLE 9

ANALYSIS OF NUMBER OF SILVER GRAINS IN NUCLEAR EMULSION IN REGIONS OVERLYING SECTIONS OF PARTICULAR AXONEMES AND KINETOSOMES IN PROTHERS AND OPISTHES OF TWO CELL LINEAGES*

Lineage	Exposure, Days	Gullet	Source	Cilia Age, Hours	Sections	Gns/Axoneme	P/O=T, %	Sections	Gns/Kinetosome	P/O=T, %
1	2	Old	Proter	≥ 14	18	$0.107 \pm .007$	51.7	18	$0.28 \pm .04$	56
		New	Opisthe	2	11	$0.207 \pm .017$		11	$0.50 \pm .13$	
2	3	Old	Proter	≥ 14	42	$0.187 \pm .008$	47.3	40	$0.40 \pm .02$	47
		New	Opisthe	2	29	$0.395 \pm .031$		13	$0.85 \pm .18$	

* Let quantity of transient protein (T) $\sim$ P and quantity of structural protein (S) $\sim$ O-P. Then ratio T:total protein on opisthe peniculi = $T/(S + T) = P/O$ and ratio S:total protein on opisthe peniculi = $S/(S + T) = (O - P)/O$. Structural protein of axonemes = 51%; structural protein of kinetosomes = 49%.

tion of subcategories for structural as well as for transient proteins in terms of their synthesis either early or late relevant to other events in the cell cycle.

Generation Time for Cell Organelles

Earlier estimates of the time required for the pre-trichocyst $\rightarrow$ trichocyst transition of about 20 minutes were based upon integration of ingrowth curves obtained from electron microscopic data.⁽¹⁾ In that study, the highest concentration of pretrichocysts appeared at the eleventh hour, about one hour before cell division. The experiments described here extend our information about the time required for the transition (organelle

generation time) as well as the time in the cell cycle at which maximum concentrations of preorganelles occur.

The existence during the cell cycle of maxima for preorganelles implies the existence of earlier maxima in output of corresponding messenger RNA. If so, then its time of synthesis should be revealed by the time for maximum blockade by actinomycin D.

Experiments performed on a population of cells with an average cell generation time of 16 hours show a delay of 4 hours when actinomycin D ($10 \mu\text{g}/\text{ml}$ for 1 hour) was applied at cell age 8 hours (Figure 29). Figure 30 shows the relationship between cell age and division delay. The cells are clearly most sensitive to the

FIG. 28.—Newly-formed two hour old gullet of an opisthe cell (sister cell to Figure 27) in which the grains represent the leucine of the structural as well as transient protein present in the newly formed axonemes and kinetosomes.

FIG. 29.—Delay in average time for cell division produced by actinomycin D. Cells were treated in antibiotic at the age of 8 hours.

FIG. 30.—The relationship between cell age and susceptibility to actinomycin D as manifest by fission delay. Average generation time in controls was 16 hours.

effects of actinomycin D when they are 6 hours old, or about 40% of the way in the cell cycle, which is precisely the time for the onset of DNA synthesis in *Paramecium*.⁽⁷⁾

Another approach to the question of maxima in the synthesis of organelles and their precursors is to measure the efficiency with which a labeled amino acid is incorporated into the organelle as a function of cell age. Cells of known age were treated in tritiated leucine for 10 minutes, followed by a two-hour postpulse interval in a chaser of nonisotopic leucine before sacrifice. The presence of tritiated leucine on the trichocyst was detected electron microscopically and autoradiographically by the methods described earlier.⁽⁵⁾ Figure 31 shows

FIG. 31.—Correlation between cell age and labeling of trichocysts illustrating the age at which incorporation of tritiated leucine into late trichocyst protein is maximal.

that the number of grains incorporated per trichocyst does not vary significantly at different cell ages, and that, therefore, the rate of amino acid incorporation into late trichocyst protein remains relatively constant throughout the cell cycle. On the other hand, the percentage of pretrichocysts available for labeling with leucine shows a decided peak at about the eighth hour, or about 70% of the way through the cell cycle. From the curve showing per cent labeled trichocysts as a function of cell age, a generation time of about half an hour for the pretrichocyst → trichocyst transition can be calculated by integration.

The following generalizations are consistent with these findings:

1. Peak concentrations of preorganelles occur at specific times in the cell cycle.
2. Organelle generation time is nearly independent of the stage of the cell cycle.
3. Some preorganelles are present in the cell at every stage in its cycle.

Measures of the stability and vulnerability to anti-metabolites of the preorganelles and of their precursors are in progress.

Patterns of Distribution of New Organelles among the Old

Earlier studies established that prior to cell division, the new ciliary corpuscles always appear anterior to the old ones and posterior to the old trichocysts⁽¹⁾ but failed to show the origin of the new kinetodesmal fibers. Recent electron micrographs of previously unobserved cell ages (0–20 minutes old) reveal new structures that have several properties of the kinetodesmal fibers and that probably represent an early stage in their development. These are shown in Figures 32–35 to be composed of microtubules, similar to those of the orthotubular system surrounding the contractile vacuole in preffission stages,⁽¹⁾ and not unlike those cytoplasmic fibrils and microtubules described by a number of authors.^(8–12) These structures, composed of microtubules of about 20 mμ diameter, appear collectively in cell cross sections as arched, or “half-moon” groupings of 5 to 8 circles (Figure 32), or as “jelly-roll” configurations of 20 or more ellipses (Figure 33) and occupy the crests of the ridge lines of the outer envelope system immediately overlying the old kinetodesmal fibers. Each microtubule appears to be composed of subunits of about 50A diameter. In longitudinal sections (Figure 35) these supra-kinetodesmal microtubules appear as slender filaments following the same pathway as the kinetodesmal fibers. That the supra-kinetodesmal microtubules are the progenitors of the kinetodesmal fibers is further suggested by the fact that they have, with only a singularly exceptional category, been found only in recently divided young cells in the very same regions in which the new fibers are, topologically, anticipated. The exceptional category is that of the heat-blockaded preffission cells in the earlier study⁽¹⁾ fixed and sectioned 30 minutes after blockade. Reexamination of these figures reveals supra-kinetodesmal microtubules in several regions of such cells, including even one published figure (Figure 7, Reference 1). Consistent with the present interpretation, these exceptional cells may be regarded to have had many, but not all, of their preorganelles and generative capacities thermally denatured; amongst some of those capacities still surviving would be that for synthesis of new microtubular elements in the supra-kinetodesmal positions.

Paratene Theory and the Shapes of Cells

These and other data suggest several new theoretical approaches to the problems of determination of the total three-dimensional form and pattern in such cells. The particularly difficult question of how two almost identi-

FIG. 32.—Transverse section of microtubules on ridge line of the outer cell envelope of a *Paramecium* at zero age. Note that the microtubules collectively form a crescent and their association with kinetodesmal fibers.

FIG. 33.—Oblique section of suprakinetodesmal microtubule on the ridge line, arranged in a "jelly-roll" configuration. Cell age is 15 min.

FIG. 34.—High resolution electron micrograph of microtubules. (Same cell as Figure 32.) Note the appearance of circular subunit structures in the microtubular wall, suggesting that each microtubule is composed of subunits 50A in diameter.

FIG. 35.—Longitudinal section of suprakinetodesmal microtubules (arrow) of a 20-min-old cell. A kinetodesmal fiber is associated with two kinetosomes and a perasomal sac (3 circular structures in a triangular formation).

cal (not complementary) structures—the daughter cells—result from the division of an equally identical progenitor cell has been discussed by many authors.^(3, 4, 13, 14)

The formulation presented below partially answers this question, recalls an earlier idea of a morphogenetic role of “organelle linkage groupings”⁽²⁾ and extends in specific terms rather than contradicts other more general “explanations” of form in terms of “mysterious and powerful fields of forces,”⁽¹⁵⁾ macrocrystallinity,⁽¹³⁾ and cytotoxis.⁽⁴⁾

The heart of the present theory is that one-dimensional linkage groupings of cell organelles in the form of polytenic circular catenaries serve as templates for a relatively macroscopic (supramolecular) replication process at the organelle complex level. These formulations shift the morphogenetic emphasis of classical ciliate cytology⁽¹⁵⁾ from the pole-to-pole meridians (“kine-

ties”) to the bands of latitude parallel to the fission furrow that we now call “paratenes.” (A glossary of terms and derivatives appropriate to paratene theory is given at the end of this paper.) An initial step is to map the cell surface by means of approximate geodesics (Figure 36). In conformance with the actual cell envelope those kineties in the stripe-contrast region do not extend from pole to pole, but terminate on a bias line prematurely in the right anterior and left posterior fields, respectively, of the outer envelope of the cell. An anterior tropical paratene can be abstracted as a circular band beginning at the stripe-contrast region, at a locus near A, and ending after a counter-clockwise circuit of the cell envelope at a locus near F (bottom, Figure 36). More posteriorly located paratenes, such as paratene “” may begin at some locus near B, and end at a locus near G. Each new or replica paratene appears anterior to its preexisting or template paratene, and viewed in the long range of many successive cell generations, the replicas tend to suffer gains at beginning (or A region) loci and losses at terminal (or F and G region) loci. Each paratene is a discontinuous one-dimensional linkage grouping, composed of about seventy loci, each locus consisting of the familiar repeat-unit of the cell envelope,⁽¹⁶⁾ the ciliary corpuscle. The relationship between kineties, paratenes, ciliary corpuscles and trichocysts is shown in Figure 37. The rule of anterior origin of ciliary corpuscles probably applies not to the whole cell, nor to its paratenes, but to the ciliary corpuscle itself, with its own orientation arbitrarily specified as follows: “left” is that direction in which the parasomal sac and kinetodesmal fiber are located. Normally then all replica paratenes are anterior to their templates. The interesting inversion reported by Beisson-Schecroun⁽¹⁷⁾ and Sonneborn⁽⁴⁾ can be mapped in terms of paratenes, and is treated as an insertion in Figure 37 represented by an inverted C. Following the rules of anterior origin, Figure 38 shows the process of paratene multiplication in four steps, the initial one being development of the new ciliary corpuscle (C) from precursors (pc) immediately anterior to the old C and posterior to old trichocysts (t). The final step is the development of trichocysts from pretrichocysts (pt) inserted between adjacent C's. Whereas the kinety increases its length by a process of irregularly spaced intercalations, it is interesting to note that the paratene (left normal side) is entirely duplicated by a method of semiconservative replication at the organelle complex level; i.e., preexisting unit organelles are conserved and do not divide, while new unit organelles develop in a topological relationship of replica to template alongside the old. The extent to which at the molecular level ancestral molecular components of the old organelles are conserved or turn over remains to be assessed, but our present evidence (also Reference 5)

FIG. 36.—A geodesic mapping of the outer envelope of *Paramecium* in terms of kineties and paratenes.

FIG. 37.—The structural basis of kineties and paratenes, and the mode of mapping a structural anomaly.

indicates a considerable degree of conservation of the essential structures. On the right side of the paratene of Figure 38 is shown the hypothetical behavior of a Beisson-Sonneborn inversion of two ciliary corpuscles, in which the rule of antecorpi is assumed to be followed. An important occurrence at step 4 is that the initial state of the paratene as a whole is not duplicated, but a new

FIG. 38.—The process of paratene multiplication in four steps. C, ciliary corpuscle; t, trichocyst; pc, preciliary corpuscle; pf, prekinetodesmal fiber; dotted lines in step 3 represent prekinetodesmal fiber.

anomaly is created; the insertion behaves as an independent paratene whose replica always appears posteriorly.

The situation represented by the normal end of the paratenes of Figure 38 is probably more appropriate for a cell in which end-to-end intercalation is the rule, such as diagrammed in Figure 39, step 4 for *Pseudocohnilembus*; ⁽¹⁸⁾ more appropriate for *Paramecium* is Figure 39, step 4', in which the bulk of the new material appears in the mid-cell fission furrow region, resulting in pauci-hemispherical addition and represented in terms of replica paratenes in Figure 40.

The usefulness of the theory of paratenes can only be appraised by realistic trial, and this has not yet been done. By inspection of its rules it appears capable of appraising the consequence to future cell generations of

FIG. 39.—The intercalational (4) and pauci-hemispherical (4') types of paratenal replication.

FIG. 40.—The relationship of replica paratenes to template paratenes near the fission furrow region.

preexisting extragenic gross irregularities of organelle networks in a given cell, predicting probabilistically not only remarkable persistence over hundreds of fissions, but also the transpositional migrations that are invariably observed and that frequently culminate in losses and concomitant normalcy.⁽¹⁹⁾

We wish to acknowledge the able assistance of Mr. David Ashbach, who as a Student Aide in our laboratory performed the experiments with actinomycin D described herein.

A GLOSSARY OF PARATENES (*para*-beside, *tene*-band)

Paratene—array of corpuscles || fission furrow in form of 1-D circular linkage grouping

Polyparateny—stacks of paratenes; at one paratene locus = kinety

Paratene multiplication—at the organelle level, semiconservative replication

Paratene properties replicated (H of template paratene) $\left\{ \begin{array}{l} \text{number} \\ \text{period} \\ \text{polarity} \end{array} \right\}$ of organelles

Paratene properties not replicated (H and/or synthesis elsewhere) $\left\{ \begin{array}{l} \text{most molecular properties} \\ \text{(exception: kd fibers?)} \end{array} \right\}$

Paratenal deletions (end losses) }
Paratenal additions (end gains) } → transpositional migration

Interparatenal recombination (fault-line displacements) → losses, gains

REFERENCES

1. Ehret, C. F. and G. de Haller. Origin, development and maturation of organelles and organelle systems of the cell surface in *Paramecium*. J. Ultrastruct. Res. Suppl. 6, 1-40 (1963).
2. Ehret, C. F. Information content and biotopology of the cell in terms of cell organelles. *Symposium on Information Theory*

- in *Biology*, ed. H. Yockey, R. Platzman and H. Quastler. Pergamon Press, London, 1958, pp. 218-229.
3. Ehret, C. F. Organelle systems and biological organization. *Science* 132, 115-123 (1960).
4. Sonneborn, T. M. The differentiation of cells. *Proc. Natl. Acad. Sci.* 51, 915-929 (1964).
5. Ehret, C. F., N. Savage and J. Alblinger. Patterns of segregation of structural elements during cell division. *Z. Zellforsch. Mikroskop. Anat.* 64(1), 129-139 (1964).
6. de Haller, G., C. F. Ehret and R. Naef. Technique d'inclusion et d'ultramicrotomie, destinée à l'étude du développement des organelles dans une cellule isolée. *Experientia* 17, 524-526 (1961).
7. Kimball, R. F., L. Vogt-Köhne and T. O. Caspersson. Quantitative cytochemical studies on *Paramecium aurelia*. *Exptl. Cell Res.* 20, 368-377 (1960).
8. Ledbetter, M. C. and K. R. Porter. Morphology of microtubules of plant cells. *Science* 144, 872-874 (1964).
9. Pease, D. C. The ultrastructure of flagellar fibrils. *J. Cell Biol.* 18, 313-326 (1963).
10. André, J. and J. P. Thiery. Mise en évidence d'une sous structure fibrillaire dans les filaments axonomatiques des flagelles. *J. Microscopie* 2, 71 (1963).
11. Pitelka, D. R. Fine structure of the silverline fibrillar systems of three tetrahymenid ciliates. *J. Protozool.* 8(1), 75-89 (1961).
12. Roth, L. E. Ciliary coordination in the protozoa. *Exptl. Cell Res. Suppl.* 5, 573-585 (1958).
13. Weiss, P. From cell to molecule. *The Molecular Control of Cellular Activity*, ed. J. M. Allen. McGraw-Hill, New York, 1962, pp. 1-72.
14. Dean, A. C. R. and C. Hinshelwood. What is heredity? *Nature* 202, 1046-1051 (1964).
15. Lwoff, A. *Problems of Morphogenesis in Ciliates*. John Wiley and Sons, Inc., New York, 1950.
16. Ehret, C. F. and E. L. Powers. The cell surface of *Paramecium*. *Intern. Rev. Cytol.* 8, 97-133 (1959).
17. Beisson-Schecroun, J. Hereditary maintenance of spontaneous and provoked variations of cortical organization in *P. aurelia*. *Excerpta Medica* 77, 18 (1964).
18. Evans, F. R. and J. O. Corliss. Morphogenesis in the hymenostome ciliate *Pseudocohnilembus persalinus* and its taxonomic and phylogenetic implications. *J. Protozool.* 11, 353-370 (1964).
19. de Haller, G. About inheritance of morphological characteristics of the cortex in *Paramecium aurelia*. *J. Protozool. Suppl.* 11, 48 (1964).

ULTRASTRUCTURAL AND GROWTH STUDIES ON SLIME MOLDS

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PURPOSE AND METHODS

The purpose of this study has been to acquire an understanding of the events involved in the transformation of a slime mold myxamoeba into a flagellate, with particular regard for such problems as the origin of kinetosomes and flagella. This report deals with the approaches, both direct and indirect, used to achieve this objective: 1) ultrastructural observations on the transformation of *Didymium nigripes* myxamoebae into

flagellates, 2) ultrastructural studies of mitochondria of the myxamoeboid and flagellate stages of *Didymium*, and 3) the establishment of *Didymium* and *Physarum cinereum* in bacteria-free or axenic cultures.

Four-day-old populations of *Didymium* myxamoebae were washed in glass-redistilled water to induce flagellation. At appropriate time intervals, cells were fixed and processed for electron microscope examination.⁽¹⁾ The methods of Nass and Nass⁽²⁾ were followed for enzym

FIG. 41. —*Didymium myxamoeba*. Nucleus (N) is typically vesicular; the nucleolus (n) consists of granular material of varying density. Favorable sections of mitochondria (M) show central core. Endoplasmic reticulum (ER) is scattered throughout the cytoplasm. Food vacuoles (FV) contain bacterial carcasses in varying degrees of digestion. Contractile vacuole (CV) also present. Note general lack of polarization.

hydrolysis studies of osmium-fixed material. Axenic cultures of myxamoebae were established through the use of an antibiotic mix and a previously described culture system.⁽³⁾

PROGRESS REPORT

Transformation of Didymium Myxamoebae

The process of flagellation is characterized by nuclear migration, establishment of polarity in an otherwise nonpolarized myxamoeba (Figure 41), and the synthesis of fibrillar systems found in association with the kinetosomes. The centrioles of the myxamoeba function

FIG. 42.—Diagrammatic representation of transformation from myxamoeba (a) to flagellate (f). Typical myxamoeba (a) grades into form which shows change in shape of nucleus (b). Nucleus of (c) elongate. Streamlining occurring in (d) with cytoplasm coming to lie behind the nucleus. This is continued in (e). Notice the centriolar complex (Cn) or centrosome and its proximity to the nucleus. Axonemes of flagella would probably form in stage (d) or (e). Note definite polarity of flagellate stage (f). Contractile vacuole (CV) denotes posterior region of developing stages.

as the kinetosomes of the flagellate; flagellar axonemes appear to form within the cytoplasm and are extruded. Figure 42 is a diagrammatic representation of the stages in the transformation of a myxamoeba into a flagellate.

Mitochondrial Ultrastructure

The mitochondria of *Didymium* are superficially similar to those of many Protozoa, with inward-projecting tubules taking the place of the cristae seen in mitochondria of higher forms (Figure 43). The mitochondrial matrix is essentially granular with a dense core (110–

180 m μ in diameter) composed of 4-m μ fibrils occupying the central region. Another type of inclusion—most often seen as a pair of electron-dense threads—is found within the tubules (Figures 44–48). Each of the threads is seen as a tightly coiled structure (helix) with a pitch ranging from 12 to 36 m μ ; in general, the shorter the pitch, the greater the electron density of the paired helices. The diameter of the helix is about 15 m μ ; the pair of helices measures about 35 to 40 m μ in diameter. These threads resemble in size and organization the helices seen in the nucleoplasm of *Amoeba* and *Pelomyxa*.^(4, 5)

Enzyme hydrolysis with ribonuclease and deoxyribonuclease suggests that the core of the mitochondrion is composed of deoxyribonucleic acid while much of the granularity of the matrix is due to ribonucleic acid. Enzyme treatment, however, appears to have no differential effect on the intratubular helices.

Axenic Cultivation

Two species of slime molds have been established in peptone-yeast extract-liver medium to which heat-killed bacteria must be added. Under these conditions, growth of *Didymium* results in approximately 7×10^6 myxamoebae/ml (from an inoculum of 10^3 cells/ml) with a generation time of 14.5 hours; growth of *P. cinereum* yields 5×10^5 myxamoebae/ml (from an inoculum of 10^3 cells/ml) with a generation time of 8.8 hours. *Didymium* remains in the myxamoeboid stage though a type of cyst forms in older cultures. *Physarum* forms plasmodia which feed on the myxamoebae, though the plasmodia do not sporulate. These cultures should greatly facilitate electron microscope autoradiography studies using labeled precursors to follow synthesis of the flagellar apparatus in transforming myxamoebae.

REFERENCES

- Schuster, F. Electron microscopy of the flagellate stage of the slime mold, *Didymium nigripes*. J. Protozool., 11 (Suppl.), 24 (1964).
- Nass, S. and M. Nass. Intramitochondrial fibers with DNA characteristics. II. Enzymatic and other hydrolytic treatments. J. Cell Biol. 19, 613–629 (1963).
- Schuster, F. Axenic cultivation of *Naegleria gruberi*. J. Protozool., 8 (Suppl.), 19 (1961).
- Pappas, G. D. Electron microscope studies on amoebae. Ann. N.Y. Acad. Sci., 78, 448–473 (1959).
- Roth, L. E., S. W. Obez and E. W. Daniels. Electron microscopic studies of mitosis in amoebae. I. *Amoeba proteus*. J. Biophys. Biochem. Cytol. 8, 207–220 (1960).

FIG. 43.—Mitochondria. Note presence of tubules. Within the matrix of the mitochondrion is the core (C). Visibility of the core depends on the orientation of the mitochondrion. Transverse sections of intratubular inclusions at (H).

FIG. 44.—Mitochondrion with unusually large number of helical structures. Note coiling.

FIG. 45.—A pair of electron-dense threads. (Pairing is difficult to make out because of the density.) Serrations on outer surface of the thread suggest tight coiling with small pitch.

FIG. 46.—Coiled thread (arrow) which was only grazed in this section; coiling can be seen particularly well.

FIG. 47.—Another example of coiling. The pitch varies in this helix.

FIG. 48.—Paired electron-dense threads. Each of the subunits shows tight coiling.

THE EFFECT OF pH, IONS AND FIXING AGENTS UPON INTRACELLULAR COMPONENTS AS SEEN WITH THE ELECTRON MICROSCOPE

Theodore N. Tahmisian, Rosemarie L. Devine, Betty Jean Wright, and Carol Christiansen

PURPOSE AND METHODS

An ideal fixing agent should stop all activity in a cell without distorting cellular components. In electron microscopy a fixing solution must yield reproducible results at all levels of organization, i.e., at the cellular, organelle, and molecular levels. Such a unique fixative does not exist. We try to approach reproducible results with respect to the cell as a whole, or with respect to a given organelle or suborganelle ultrastructure. An ideal fixative for one or several of the cellular components is achieved in various ways. The choice of a fixing agent (proper osmolality, specific ionic species mixed with the fixative, a particular buffer, and the hydrogen ion concentration of the solution) aids in morphological reproducibility in a fixed cell.

Fixing solutions used to produce a fine structure in biological material for electron microscopy fall into two classes: oxidants and reductants. Both the oxidants and reductants act as poisons by stopping, irreversibly, the biological activity of the cell. The most popular fixing solution in electron microscopy, because it produces a very fine grain without leaching of cellular proteins, is one that contains osmium as the active agent. Potassium permanganate, dichromate, aldehydes, alcohols, acetone, and heat or cold are some of the other fixatives in use in order of their popularity. Specific fixing agents have unique effects on cellular components. KMnO_4 enhances membrane fixation, $\text{K}_2\text{Cr}_2\text{O}_7$ acts more readily on nucleoproteins. The aldehydes displace some water and reduce the oxidized state of the living cell irreversibly.

The oxidizing fixatives contain metal ions. The metal ion is reduced to a metal at the site of unsaturated portions of a molecule composing the tissue. Thus a metallic soap or a metalloproteinate is formed depending on whether a triglyceride or protein molecule is oxidized by the metal. Because double bonds are not distributed uniformly throughout a cell, a differential opacity for electrons traversing the cell is obtained when a heavy metal ion is reduced to a metal at the site of the double bond. The absorption of electrons emitted from the electron microscope gun by these metals renders contrast which can be photographed or studied with the aid of a phosphorescent screen.

Fixing solutions having metal ions poison the cellular organic material so that tissues can not be subjected to enzymolysis. In order to carry out enzymatic analysis of

the tissue by enzymatic removal of specific substrates, the tissue is fixed with a nonmetallic organic chemical. For this purpose formaldehyde, glutaraldehyde, acetone or alcohol is used. When enzymatically treated tissue sections are compared with untreated controls the loci of specific proteins in the organelles can be determined.

Cells fixed with formaldehyde or glutaraldehyde do not show contrast in an electron beam; therefore, further reduction of these tissues is accomplished by the uptake of osmium, uranyl ions or other heavy elements. Thus, the fixed and digested tissue is stained for contrast.

It is not enough to fix a cell. The cell must be fixed so that it is not distorted with respect to its living counterpart. Several factors play a role in fixing a cell where the topology and morphology of organelles are not altered. Some conditions affecting the above mentioned changes are isotonicity, ionic species, pH, and developmental zero temperature. To study life processes a living cell should be observed. To date this is not possible with the electron microscope. The next procedure is to study the point a cell has reached at the time it is fixed. Fixing usually denatures proteins irreversibly, but after organic fixing, enzymes will digest specific substrates from the cell and some organelles. Furthermore, protein can be reconstituted at the macromolecular level. Therefore, it is important to know whether an organelle was in a gel or sol state when fixed, or whether it was at its isoelectric point. It must be known whether swelling or shrinking took place.

In a previous report we discussed the effect of fixing cells in a fixative which is normal saline equivalent (NSE) with respect to a specific cell or tissue.⁽¹⁾ Under these conditions distortion caused by swelling or shrinking was reproducibly controlled. Because the isotonicity of a fixing solution can not be determined without killing the cell, the freezing point depression apparatus was used to determine the ionic strength of the fixative. The fixative then was adjusted to be equivalent in ionic strength to physiological saline for that cell.

Our present problem is to fix cells under NSE conditions but with buffers ranging from pH 3 to pH 11. We hope to see the effect of fixation at and away from the isoelectric point of a protein. Since a number of proteins are present in a cell, those organelles with a preponderance of one or another protein should have an altered morphology dependent upon the pH of the fixative.

PROGRESS REPORT

Over 100 experiments and tissue embeddings were made on grasshopper testis to study results of pH, salting out, specific fixative effect, ionic species of the carrier salt, all under osmotically NSE conditions. Several effects were noticed. It is beyond the scope of this report to cover all the variations studied. We shall report some of the more striking phenomena.

It appears that the NSE fixing solutions must have a combination of Na^+ , K^+ , Ca^{++} , and Mg^{++} ions as the

carrier salt for the fixing agent such as osmium or permanganate. If only Na^+ is used the cell appears fine-grained. K^+ alone causes a more granular appearing cell. The granularity is greater with Ca^{++} or Mg^{++} . A combination of Na^+ , K^+ , and Ca^{++} with a ratio of 3:2:1 as the carrier salt appears to yield the least distortion and protein loss from the specimen (Figures 49-51).

NSE fixing solutions have different effects on the appearance of the cell. KMnO_4 enhances the appearance of membranes as reported by Luft.⁽²⁾ This we confirm. But

FIG. 49.—Late spermatid of grasshopper, fixed in $\text{NSE-OsO}_4 + \text{KMnO}_4$, 1%+1.3% respectively, with a defined ionic species provided by NaCl , KCl , and CaCl_2 at a ratio of 2-1/2:2:1, unbuffered; the ultrastructure of the membranes is well resolved as an array of discrete units. This is in contrast to KMnO_4 -alone-fixed material where membranes are resolved as a thin continuous line. The plasma membrane (PM), nuclear envelope (NE) as well as the centriolar adjunct (CA) are indicated. Stained with uranyl acetate for 1 hour after sectioning.

FIG. 50.—Grasshopper spermatid fixed in NSE-OsO_4 , + KMnO_4 , 1% + 1.3%, respectively, with the same ratio of NaCl , KCl , and CaCl_2 as in Figure 49, and treated in the same manner. The over-all fixation has a slight granularity, but all organelles are well defined. The Nebenkern (NB) contains cristae with double membranes; the nuclear envelope (NE) and Golgi (G) are typical; of particular interest are ribonucleo-protein (RNP) granules or rosettes in dark clusters throughout the cytoplasm (R) and in the nucleus (N).

FIG. 51.—Several late spermatids of grasshoppers cut either in cross section through the middle piece of the tail or longitudinally through the middle piece and the nucleus. Fixed and treated as Figure 49. The component parts of the flagellum (TF) are well preserved as is the chromatin (CS) of the elongating nucleus. The plasma membrane (PM) of the maturing spermatid differentiates to a complex structure, details of which can be seen as a periodic pattern. Other fixatives have failed to retain these details.

FIG. 52.—Grasshopper spermatid fixed in NSE-KMnO₄ at pH 6.0, sectioned, then stained with lead citrate. A most pleasant appearing fine grained fixation. The cell membrane (CM) is a fine continuous line. The Golgi (G) lamellae, the proacrosome (PA), the Nebenkern (NB) are well resolved. The nucleus (N) and the nuclear envelope (NE) are delicately fixed. The endoplasmic reticulum (ER) is well fixed but no ribosomes are present on it unlike that seen with OsO₄ + KMnO₄ fixed material. The intercellular space (IS) is well fixed and it has continuity with the cell cytoplasm as seen at the cell furrow (CF).

FIG. 53.—Grasshopper spermatocyte fixed in $\text{NSE-OsO}_4 + \text{KMnO}_4$, 1% + 1.3%, respectively, at pH6.7. The section is unstained. The fixative has very high affinity for RNP material, both in the nucleus (N) and in the cytoplasm. The nuclear envelope (NE) appears as a strand of dots with no resolution for its double membrane nature as seen in Figure 52. The endoplasmic reticulum is not resolved but the ribosomes (R) that adhere to the endoplasmic reticulum show darkly stained and also suggest the unseen loci of the reticular membranes.

FIG. 54.—*Helix aspersa* (snail) sperm tail cross section, fixed in NSE-OsO₄, phosphate buffered at pH 7.4. Sections stained with lead citrate. The normal physiological buffer for snail is pH 8.4. At a lower pH, indicated above, the fixation of all components is excellent. The Golgi network (GN) and the mitochondrial elementary particles (EP) of the sperm tail middle piece are resolved in sectioned material. The elementary particles are composed of a knoblike head, a cylindrical stem, and a base. The particle arrangement shows a periodicity of 150 Å from center to center.

we also notice that in KMnO_4 -fixed cells RNA granules on the endoplasmic reticulum are not apparent even after postosmication, or uranium or lead staining. The nuclear DNA appears washed out, and usually the mitochondria are swollen, whereas the nucleus is shrunken, even under NSE conditions (Figure 52). Cells fixed in NSE- OsO_4 show RNA, DNA, and centrioles well fixed. The fixation is not as fine grained as that obtained with KMnO_4 , but the distortion of organelles is absent. The membranes are not as well defined as seen with KMnO_4 fixation. The Golgi are well fixed with either KMnO_4 or with osmium.

An NSE combination of OsO_4 and KMnO_4 fails to resolve the membranous portion of the ER but appears to have a high affinity for RNA granules of the ER as well as RNA and DNA of the nucleus (Figure 53). The RNA wherever found appears very darkly stained. The ultrastructure of the mitochondrial membranes is discerned better with osmium fixation at a more acid pH relative to the pH of the physiological saline. For grasshoppers, a pH 6.0 solution appears optimum but for *Helix aspersa*, which has a physiological pH of 8.4, a fixative pH of 7 appears to be optimum with respect to the mitochondrial elementary particles. These particles, as described by Fernandez-Moran,⁽³⁻⁵⁾ Green,⁽⁶⁾ Parsons,⁽⁷⁾ and Stoeckenius,⁽⁸⁾ are 90 Å at the head, 40 Å at the stem, and 150 Å apart (Figure 54).

To date, we have studied pH in conjunction with fixatives within a range of 5.5–8.8. Optima for tissues of the grasshopper is a pH of 6.0, for mammals a pH of 7.4, and for *Helix* a pH of 7. We are investigating the more acid and more alkaline ranges which are not within the physiological range. It may be possible to show organelle protein composition by fixing cells at or away from the isoelectric point of the protein.

REFERENCES

1. Tahmisian, T. N. Use of the freezing point method to adjust the tonicity of fixing solutions. *J. Ultrastruct. Res.* **10**, 182–188 (1964).
2. Luft, J. H. Permanganate—a new fixative for electron microscopy. *J. Biophys. Biochem. Cytol.* **2**, 799–802 (1956).
3. Fernandez-Moran, H. Subunit organization of mitochondrial membranes. *Science* **140**, 381 (1963).
4. Fernandez-Moran, H., T. Oda, P. V. Blair and D. E. Green. A macromolecular repeating unit of mitochondrial structure and function. *J. Cell Biol.* **22**(1), 63–100 (1964).
5. Fernandez-Moran, H. New approaches in correlative studies of biological ultrastructure by high-resolution electron microscopy. *J. Roy. Microscop. Soc.* **83**, 183–195 (1964).
6. Green, D. E., P. V. Blair and T. Oda. Isolation and characterization of the unit of electron transfer in heart mitochondria. *Science* **140**, 382 (1963).
7. Parsons, D. F. Mitochondrial structure: Two types of subunits on negatively stained mitochondrial membranes. *Science* **140**, 985–987 (1963).
8. Stoeckenius, W. Some observations on negatively stained mitochondria. *J. Cell Biol.* **17**, 443–454 (1963).

STUDIES CONCERNING THE DEVELOPMENT AND CELLULAR REACTIONS OF CONNECTIVE TISSUE USING THE SPONGE METHOD

F. Wassermann

PURPOSE AND METHODS

The object of these experiments is to obtain information about the reactions of connective tissue to chemical agents, hormones, and ionizing radiation under controlled experimental conditions.

Sponges of polyvinyl plastic resin ("Ivalon" sponges) in the form of 5-mm cubes are inserted under aseptic conditions either into the space between the stumps of severed tendons or into the intact subcutaneous connective tissue or into the subperitoneal tissue, using rats weighing about 200 g. The inserted material becomes gradually occupied by highly vascularized young connective tissue. At certain intervals the sponges are removed, and parts of them are prepared for microscopic and electron microscopic examination. These tissue samples serve as controls for further experimentation. The insertion of the sponges at different sites is done in order to recognize local factors which may influence the

growth and the cell population of the young connective tissue.

The basic data were obtained in 1963–1964. Further experiments are now being made, exposing the site of insertion of sponges to local X radiation to study the radiation effects on the tissue. Only sponges inserted into the severed tendons have been used so far; subcutaneously implanted sponges will also be used. Tissue was taken for examination at various intervals after insertion and after irradiation. Other experiments are being made with sponges as carriers containing pellets of agents whose effect on the tissue is to be studied.

PROGRESS REPORT

In 1963–1964 numerous experiments had to be made in order to work out the suitable surgical procedures and techniques, especially for the electron microscopic examination of the tissues. Thus the background for the above indicated experiments has been established.

In the irradiation experiments so far, 1000 to 3000 R were applied to the sites of sponge implantation. The study of the material obtained from these experimental animals is in progress. Preliminary examination showed a remarkable radioresistance of the fibroblasts. Although the formation of fibrils was delayed for about two days after irradiation, the tissue recovered completely after about five days. Further experiments are under way to achieve more outspoken effects with doses higher than 3000 R.

RADIOAUTOGRAPHIC ELECTRON MICROSCOPIC STUDY OF THE TRANSPORT OF LIPIDS USING C¹⁴-LABELED FATTY ACIDS

F. Wassermann and Walter E. Kisieleski

PURPOSE AND METHODS

In a previous electron microscopic study by F. Wassermann and Thomas F. McDonald⁽¹⁾ the pathways of lipids between the blood and the fat cells have been traced with osmic acid as a marker. It was recognized that a more exact method is needed to localize the fatty acids, especially where they are too highly dispersed to be demonstrable by osmic acid staining. The given method is radioautography.

Adipose tissue is taken from rats which received C¹⁴-labeled fatty acids by intravenous injection after a period of fat-free diet. The time interval between the injection and the excising of the tissue is varied from one to 24 hours. The tissues are fixed, embedded in plastic and sectioned for electron microscopic examinations. The ultrathin sections are covered with a thin film

Pilot experiments with sponges containing pellets of hydrocortisone were made in 1964. The tissues are now under examination.

In our recent experiments sponges were implanted in the shielded and the irradiated parts of the animals. In other experiments sponges with and without the agents to be tested were implanted in the same animal. In this way control material and test material were obtained from the same animal at the time when the experimental reactions were taking place.

of photographic emulsion and kept in the dark for exposure. After exposure and photographic developing, dark grains will mark the sites of fatty acids along the pathway of lipids in transport and also within the cell.

PROGRESS REPORT

Adipose tissue is notoriously one of the most difficult objects in electron microscopy, and radioautography became applicable to electron microscopy only very recently. Our study is therefore still in a preliminary stage mainly concerned with technical problems.

REFERENCE

1. Wassermann, F. and T. F. McDonald. Electron microscopic study of adipose tissue (fat organs) with special reference to the transport of lipids between blood and fat cells. *Z. Zellforsch. Mikroskop. Anat.* 59, 326-357 (1963).

FINE STRUCTURE OF THE OSTEOCYTE CAPSULE

F. Wassermann and J. A. Yaeger

PURPOSE AND METHODS

For a better understanding of the pathway of transport of material between the blood and the bone tissue, the fine structure of the walls of the lacunae in bone has been studied with the electron microscope.

Small pieces of bone from the femora of dogs, guinea pigs, and rats of various age groups were fixed in a series of fixing agents available for electron microscopy. The bone tissue was either used in the calcified form or was decalcified before embedding in plastic. The undecalcified embedded material could be sectioned with the diamond knife. The use of different fixing agents and of both decalcified and undecalcified bone made it possible to carry out this study on a much broader basis than has been done before.

PROGRESS REPORT

The fine structure of the bone cell was demonstrated,

and variations in structure could be related to function and age of the cells. The material in the immediate surrounding of the bone cell was recognized as collagen fibril-containing tissue which makes it certain that the "capsule" of the bone cell is of the nature of connective tissue and that the transport of material in bone is mediated by the same mucopolysaccharide-containing ground substance that is the medium of transport throughout the body. It was found that a border zone exists between the uncalcified capsule of the bone cell and the bulk of the calcified matrix. In this zone the bone crystals are less orderly arranged than in the bone matrix proper and seem to represent, as "coastal" crystals, the metabolically more labile minerals in bone. This study has been brought to conclusion in 1964 and a paper with the above title is ready for publication.

ENVIRONMENTAL RADIATION STUDIES

Philip F. Gustafson and Sarmukh S. Brar

PURPOSE AND METHODS

The purpose of these studies is to investigate the levels of fission product and natural radioactivity present in the Chicago environment. Toward this end, the concentrations of various radionuclides have been determined in surface air, soil, foodstuffs, human bone, and to a limited extent human tissue collected in the Chicago area. These data all or in part have a three-fold use in the study of: 1) atmospheric transport of nuclear test debris and its deposition, 2) movement of specific fission products through the biosphere and ultimately to man, and 3) the radiological importance of fission products relative to natural radioactivity.

Almost exclusively the radioactive analyses are made by gamma-ray spectrometry using large NaI crystals and low background counting facilities. The preparation of samples prior to gamma-ray counting is minimal, and for the most part no ashing or chemical processing is involved. Bulk amounts of sample (1 to 2 kg) are counted when available. Analysis of the spectral data is carried out by computer. Routine analyses are made for the following fission products: Cs^{137} , Ce^{141} , ^{144}Ru , ^{103}Ru , ^{125}Sb , ^{95}Zr - ^{95}Nb , Ba^{140} - La^{140} . In addition Mn^{54} produced by activation, and Be^7 arising from cosmic ray interactions in the atmosphere are also determined when appropriate.

PROGRESS REPORT

Global transport of nuclear test debris proceeds via the atmosphere. Hence, the first evidence of new debris appears in surface air within a few weeks after a test has been made. During and for some time after an atmospheric test series the concentration of fission products may remain at elevated levels and may also be quite variable with time. In addition, it has been observed repeatedly that maximal concentrations of fission products occur in surface air during the spring months. This is most pronounced in the case of long-lived fission nuclides, such as Cs^{137} , and is likewise true for naturally occurring Be^7 . The magnitude of the spring maximum is

closely related to the amount of radioactivity present in the lower portions of the stratosphere. The time of appearance of the maximum shifted from April-May seen in 1955 through 1960 to May-June in 1961, June-July in both 1962 and 1963, and back to May-June in 1964. A similar shift has been noted at a number of other sites in the northern hemisphere, and may reflect a change in atmospheric circulation or other meteorological phenomena affecting a wide area. It has also been found that a significant portion of the activity seen in surface air during the last 4 years originated in the high stratosphere. Prior to the resumption of testing in 1961, the highest concentration of Cs^{137} observed in surface air at Argonne was 0.035 pCi/cubic meter in the spring of 1959. The maximum concentrations measured since that time were 0.078, 0.165, and 0.104 pCi/cubic meter in 1962, 1963, and 1964, respectively. These values maintain a rather constant relationship with the corresponding stratospheric inventory. By means of specific tracers, namely Rh^{102} and Mn^{54} , as well as through the use of activity ratios between certain fission products, it has been possible to partition the total Cs^{137} observed in air into that arising from various sources (i.e., various test series). On this basis some 70-80% of the debris seen during 1962 through the present appeared to have arisen from tests conducted at arctic latitudes. Utilizing past data, it is possible to predict with some certainty the magnitude of the coming spring maximum. For example, the predicted value for 1964 was 0.11p Ci/cubic meter, which was close to the observed concentration of 0.104 pCi/cubic meter.

The removal of airborne fission debris proceeds primarily by precipitation (80 to 90% of the total deposition comes via precipitation), with dry deposition being of secondary importance. The high air concentrations in the spring coupled with the relatively greater precipitation during this season results in the bulk of the annual deposition coming during the spring and early summer. During and shortly after times of testing there is a pronounced accumulation of fission debris on the ground,

particularly of the shorter-lived fission products. As the time elapsed since testing increases, the rate of accumulation decreases, approaching the rate of loss due to radioactive decay. Finally the rate of decay exceeds that of deposition, and the total present on the ground diminishes with time. Because of their long radioactive half-lives, Cs^{137} and Sr^{90} tend to increase, albeit slowly, even during times of slight deposition. Hence, the Cs^{137} , for example, present on the ground today represents a summation from all past tests. The radioactivity present on the ground gives rise to an external dose of gamma radiation and is also available, in part at least, for plant and animal uptake. The accumulation of Cs^{137} in local soils up to mid-1961 amounted to approximately 200 mCi/mi². The deposition during 1962 was 38 mCi/mi², in 1963 78 mCi/mi², and through August 1964 the deposition has been 39 mCi/mi². Thus, the total now present on the ground is approximately 330 mCi/mi² (account being taken for the decay of older Cs^{137}). Using a $\text{Cs}^{137}/\text{Sr}^{90}$ activity ratio of 2:1 implies that the corresponding Sr^{90} deposition should be some 165 mCi/mi². Due to the half-life effect discussed above, the greatest accumulation of the shorter-lived radionuclides occurred during the summer of 1963. Correspondingly the highest external open field dose rate also prevailed at this time and amounted to some 7.5 $\mu\text{R}/\text{hour}$ in June 1963. The external dose rate due to uranium, thorium, and potassium has been found to be 8.0 $\mu\text{R}/\text{hour}$ on the Argonne site, and an additional 4.4 $\mu\text{R}/\text{hour}$ has been measured for cosmic radiation. Thus, in June 1963 the fission product dose rate was essentially equal to the natural terrestrial dose rate, and amounted to an increase in total exposure of some 60%. This was roughly twice the fission dose rate seen during the peak months of 1962, and the fission product dose rate during June and July 1964 amounted to $\sim 3.0 \mu\text{R}/\text{hour}$.

Studies of the Cs^{137} content of grains have been conducted for a number of years and have clearly indicated higher concentrations of Cs^{137} in the fruit during years of high deposition. This suggests that incorporation occurs via foliar uptake or ready utilization of fresh fallout by the roots rather than depending primarily upon total Cs^{137} present in the soil. Thus within the year of deposition one sees increases in the Cs^{137} content of flour and other grain products. Collections of foods comprising a standard diet have been made in Chicago since April 1961. These have been part of a cooperative effort with the Health and Safety Laboratory in New York as part of the Tri-City Sampling Program. A portion of the foods collected is sent to New York and the remainder is kept here for Cs^{137} analysis. The principal sources of Cs^{137} in the diet have been found to be milk, grain products, and meat. The computed daily intake of Cs^{137}

has risen from a value of ~ 50 pCi/day in April and July 1961 to a high of 240 pCi/day in January 1964 (a five-fold increase). The April and July 1964 levels were 224 and 187 pCi/day, respectively, indicating a slow decrease in dietary level. The biological half-life of Cs^{137} in the human body is relatively short, reported to be on the order of 100 days in adults. Hence, differences in the Cs^{137} intake should be matched quite rapidly by corresponding changes in the body burden of this nuclide in humans. Indeed, the whole-body measurements of Cs^{137} made at Argonne show an increase by a factor of 4 between mid-1961 and early 1964, and by April 1964 have increased by a factor of 5 over those seen in 1961.* Because of the biological half-time a finite lag would be expected between diet and body levels.

It has also been of interest to determine the Cs^{137} content of human rib bone and muscle in the same individual. Measurements made on rib bone in 1961 before the resumption of nuclear testing showed that rib contained concentrations of Cs^{137} comparable or somewhat greater than those found in muscle. In more recent bone sampling, the Cs^{137} increased by approximately 20% in 1962, and a 40–50% increase over 1961 was seen in 1963. This is in contrast to the marked increase in total body levels. Hence, the current situation is one in which the bone levels are exceeded by the muscle values by roughly an order of magnitude. This one would expect if the Cs^{137} in bone is actually tightly bound. The biologic half-time of bone seekers such as Sr^{90} is on the order of many years, and therefore, changes in intake will produce a minimal change in bone concentration, particularly in adults. The radiological importance of Cs^{137} as a source of bone exposure is now due primarily to Cs^{137} in the surrounding tissue, and that in bone itself will become of relative importance only after the whole-body levels have diminished. It should be stated, however, that the dose to bone from total body Cs^{137} exceeds at present that from Sr^{90} but will diminish more rapidly in the absence of weapons tests than will the Sr^{90} .

CONCLUSIONS

The principal effect of fission products in the environment during 1963 has been to increase the open field dose rate by ~ 35 mR/year over and above that of 108 mR/year due to natural radioactivity and cosmic radiation. A shielding factor of 2 has been found appropriate for the attenuation of fission product dose in that little contamination from this source is found indoors. Hence the effective dose increment is more like 15 to 20 mR/year and amounted to a 15 to 20% increase in external exposure to the population in this area. The dose from internal sources, principally K^{40} , and to a

* The whole-body data were kindly supplied by Dr. C. E. Miller of the Health Division.

lesser extent C^{14} and radium, has been estimated to be 20 mR/year. The additional exposure from Cs^{137} and Sr^{90} in the body will amount to a few mR/year. Hence, if we take 130 mR/year as the average total dose to the individual from natural sources, both internal and external, then the increase in exposure due to fission products was some 15 to 20% in 1963. The exposure

rate thus far in 1964 has proceeded at a rate roughly 40% of that in 1963 for external sources. Presumably the internal exposure will remain roughly the same or go up slightly due to the increase in Cs^{137} burden mentioned above. Hence, the total exposure increment in 1964 may be presumed to be of the order of 10 to 15% above that due to natural sources.

BALLOON-BORNE GAMMA-RAY SPECTROSCOPY PROGRAM

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PURPOSE AND METHODS

The purpose of this program is to locate radioactive nuclear test debris in the upper atmosphere by means of a balloon-borne scintillation detector system (designated GASP). A flight using such a device determines the distribution and, to a degree, the character of the radioactivity involved. The detector consisted of a cylindrical $CsI(Tl)$ crystal $1\frac{1}{4}$ in. in diameter by $1\frac{3}{4}$ in. long enclosed in scintillating plastic, forming a phoswich for the detection of gamma rays and the rejection of events due to the passage of charged particles through the system. Charged particles of energy less than 0.25 MeV could not be reliably rejected; hence, the lowest photon energy examined was 0.3 MeV and the range extended up to 4.0 MeV. Events due to the interaction of gamma rays in the CsI crystal were transmitted by a standard U.S. Weather Bureau 1680-Mc radiosonde transmitter, the carrier of which was turned off for a time interval proportional to the pulse height in CsI . The signal was received by a Signal Corps GMD-1A receiver-tracking antenna system. The time-modulated signal was then converted back into pulse-amplitude form. Pulse-height data were collected either on a multichannel analyzer or on magnetic tape. In addition count rate information was recorded on a strip chart recorder.

During October 1963 a series of experimental flights was made by piggy-backing equipment on large balloons flown by the U.S. Air Force from Goodfellow Air Force Base at San Angelo, Texas. Seven flights were made in all, and the following instruments were flown: 1) a large phoswich (3-in. diameter by 4-in. long CsI crystal) to study increased sensitivity; 2) a standard GASP with an internal temperature probe to measure operating temperatures and their effect upon system stability; 3) a standard GASP which switched its mode of operation between response to photons only (the usual mode of operation) and response to photons plus charged particles (no charged particle rejection); 4) a GIGI (gross-

gamma device designed and built by AEC Health and Safety Laboratory in New York) flight in which gross count rate information was simultaneously transmitted and recorded on a recorder flown with the instrument.

The prime purpose of all of these flights was to obtain a fairly sizable air sample at a specified altitude. This involved having the balloon hover for several hours during the course of sample collection. The GASP is normally powered by water-activated batteries, the useful lifetime of which is $2\frac{1}{2}$ to 3 hours. In order to provide power for 4 to 8 hours it was necessary to use dry batteries. This proved to be a source of difficulty in that the dry batteries tended to deliver substantially lower voltage at the low temperatures encountered during the night portion of the flights, the balloons being launched well before sunrise in most cases. Fortunately the effect of low temperature on the power supply did not preclude collection of useful data during much of ascent as well as during the latter portions of the hovering phase.

PROGRESS REPORT

The switching GASP flight indicated that although there was an additional counting rate due to charged particles (approximately 15% increase) this in no way impaired the quality of the spectra obtained. Hence, the new GASP design eliminates the charged particle rejection feature and reduces the unit cost considerably and results in some reduction in weight. The temperature stability should also be improved by the circuit simplification. The large phoswich proved to be considerably more sensitive than anticipated and adequately demonstrated the feasibility of flying a larger detector. The temperature investigations indicated that with the low temperatures encountered at night the gain changed sufficiently to alter the spectra seriously. The change in gain was observed by placing a small Na^{22} source near the detector during flight, providing a continuous calibration. To correct for temperature effects altering the spectra in subsequent flights it has become standard practice to include a Na^{22} source in all flight

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packages. There was also an indication that sunlight was an important source of heat for maintaining a more suitable operating environment. A quantitative measure of solar heating was not possible because of the differences in flight patterns. The GIGI flight showed that there was no essential difference between transmitted and directly recorded data throughout the entire flight. This was an important verification as the GIGI is in use at a number of sites, and such a comparison had not been made previously.

During the period November 1963 to May 1964 the balloon program was at a standstill as to new developments due to lack of operating funds. However, much of the earlier data, particularly from the GIGI stations were more thoroughly examined during this time. Flights on a monthly basis were continued at Barrow, Alaska, and the station at Antofagasta, Chile, made flights each quarter. The Alaskan results have shown the continuing presence of fission debris within the middle and high stratosphere, with some downward movement evident during the winter and spring.

In June 1964 two GASP flights were made from Chanute Air Force Base, Illinois, for the purpose of col-

lecting more definitive data concerning the effect of solar heating. The first flight was started long enough after sundown so that the instrument never rose into sunlight. The second flight was made during the morning hours well after sunrise. The ambient temperature profiles were almost identical during the two flights. Thus the differences in internal temperature may be attributed solely to the presence or lack of sunlight. During the night flight internal temperatures dropped slightly below 0° C (minus 5° C), whereas the lowest temperature recorded during the daylight flight was ~10° C. A uniform shift in gain as a function of temperature was noted. This shift has been compensated for through the use of a computer program applied to the magnetic tape data.

A more sophisticated telemetry system using FM-FM was investigated during September 1964. The probable increase in stability to be achieved with this system compared with the present telemetry does not appear to warrant the extra expense. Owing to the high unit cost and the expense involved in building a suitable ground facility the FM-FM telemetry system is not being considered at the present time.

AN INVESTIGATION OF COPPER AND OTHER SELECTED ELEMENTS IN SOILS UTILIZING GAMMA RADIATIONS INDUCED BY THERMAL NEUTRON ACTIVATION

Jerry R. Kline

PURPOSE AND METHODS

Plants growing in nature must derive their mineral nutrient requirements from the soils in which they are grown. These soils generally consist of complex mixtures of silicate and other minerals in various stages of degradation by weathering, intermixed with partially decomposed organic material. A problem of interest in soil chemistry relates to the mechanism by which mineral nutrients are delivered to root surfaces. It seems unlikely that the soil solution is similar to a nutrient culture solution since the processes of weathering and leaching over geologic spans of time would remove plant nutrients irreversibly from the soil via the ground water. It is equally unlikely that the degradation of silicate minerals is of immediate significance in plant nutrition since these minerals are not significantly altered in time intervals corresponding to the life spans of most plants. Much of the interest in soil chemistry then centers on the chemical forms of plant nutrients in soils which are available for use by the plants. Two forms which are of importance in this regard are those sorbed on the surfaces of colloidal secondary silicate minerals

known as "clays" and those which have reacted by any of several means with the partially degraded organic matter.

The study being reported here was the subject of a Ph.D. thesis investigation by the author and was carried out at Argonne under a predoctoral thesis appointment. The primary objective of the study was to measure the chemical forms of copper in a variety of soil samples and to attempt to assess their availability to plants, using neutron activation to label the soils prior to the analysis.

Six soil samples from several locations in Minnesota were selected for analysis. These samples were chosen to represent a variety of properties including the conditions of origin. One sample was taken from loessial (wind blown) deposits, three were from lacustrine sediments (ancient glacial lake bottoms), and two were from areas of glacial till deposition. The soils were subject to chemical fractionation after neutron irradiation in the large rabbit facility of the CP5 reactor at a flux of 2×10^{13} n/cm²/sec for periods of 5 to 10 minutes.

Three fractions of the total soil copper were obtained.

The first fraction which was thought to represent that copper most easily available to plants was obtained by a process of self-diffusion. In these experiments the soils were allowed to equilibrate with dilute solutions of non-radioactive CuSO_4 . The release of copper-64 as a function of time was then observed. The self-diffusion experiments are based on the concept of dynamic equilibrium. When a labeled copper atom from the soil ionizes into a surrounding aqueous medium it would be expected that the vacancy thus created would be filled by one of the far more numerous unlabeled copper atoms of the liquid medium. Thus that fraction of soil copper which is in the most freely ionizable form appears most rapidly in the extracting liquid, and it is this form which is of greatest significance in the mineral nutrition of plants.

When the diffusible fraction had been removed the soils were extracted in dilute copper sulfate solutions which were 0.1 *N* in HCl. This fraction may be representative of more or less easily degradable minerals in soils and could be of significance in future plant nutrition due to release by weathering.

The third fraction consisted of the residual copper after the first two fractions had been removed. This form was suggested to be derived from the silicate minerals of the soil and is probably a highly stable form of little significance in plant nutrition.

Separation of copper from the soil solutions was done by sulfide precipitation from acid medium using thioacetamide as a generator of H_2S . A single precipitation resulted in sufficient removal of other radioisotopes so that quantitative results could be obtained by gamma-ray spectrum analysis. The chief contaminants of the sulfide precipitates were traces of sodium-24 and arsenic-76. Corrections for sodium were made by spectrum stripping using ratios obtained from standards. Corrections for arsenic were made using a combination of chemical and spectral techniques.

PROGRESS REPORT

The total copper in soils ranged from 2.7 to 17.6 ppm in the surface and from 2.3 to 17.9 ppm in the sub-surface. In general there was no indication that copper had been leached downward in the soils by the weathering processes.

The fractionation of soil copper indicated that from 0.8 to 11.1 ppm could be removed in the self-diffusion process. These amounts were a dominant proportion of the total copper and were roughly correlated with the total. The diffusible copper was generally lower in sub-soils than in the corresponding surface soil.

Kinetic studies indicated that release of copper from soils was rapid and took place at similar rates for all soils. Equilibrium levels of copper release were generally

attained within 20 minutes from the initiation of the extraction.

The rate of release of copper from soils was not dependent upon the concentration of the nonradioactive extracting solutions. The amount of soil copper released at equilibrium was concentration dependent. More copper was released from soils by 6×10^{-3} *M* CuSO_4 than by 2×10^{-3} *M*; however, with only two exceptions, 19×10^{-3} *M* solutions did not result in increased copper release over the 6×10^{-3} *M* solution.

The organic material of soils is known to be extractable in large part in solutions of NaOH. Such extractions made on irradiated soils resulted in removal of copper in amounts approximating those removed by self-diffusion. When the diffusible copper was first removed, NaOH extractions obtained only insignificant amounts of copper. The results may indicate that a large part of the freely diffusible copper in these samples is organically bound, and only small amounts are held as exchangeable ions on clay surfaces.

Further fractionation of the soil copper revealed significant differences in the more stable forms between two groups of soils. In those soils which contained free CaCO_3 the acid soluble copper formed from 25 to 40% of the total, while the residual copper constituted less than 10% of the total. In soils which contained no free CaCO_3 the acid soluble copper was a less dominant proportion of the total and the residual was a more significant proportion of the total.

Determination of copper in plants grown on these soils under controlled conditions indicated that the amount of this element taken up was independent of the amount found in any of the soil fractions.

Other experiments consisted of observing the gamma-ray spectra of chemically unaltered neutron irradiated soils. Sodium-24 completely dominated the early spectra, and no isotopes of shorter half-life could be observed when the indicated irradiation time and flux were used. When sodium-24 had decayed sufficiently, several longer-lived isotopes were observed. These were: lanthanum-140, scandium-46, cobalt-60, chromium-51, iron-59, and cesium-134. Quantitative determinations were obtained for these elements with the exception of cesium.

The qualitative structure of the gamma-ray spectra of soils was very similar for 5 of the samples observed in spite of the fact that the samples varied widely in their conditions of origin and were obtained from locations sometimes more than 400 miles apart.

CONCLUSIONS

1. Neutron activation has value not only for analyses of materials for the total of a trace constituent

but also for chemical fractionation of a trace constituent. Yet to be determined is the degree of alteration of the sample by the neutron irradiation itself.

2. The "available" copper in the soils examined was a dominant proportion of the total and existed in amounts in excess of plant requirements in all soils.

3. The "available" copper in soils may exist dominantly in organic combination rather than as surface adsorbed ions on clay minerals.

4. The bulk of nondiffusible copper may exist as impurities in CaCO_3 crystals in soils which contain this

mineral. In soils which are free of this mineral nondiffusible copper may exist in greater quantity as impurities in the more stable silicate mineral structures.

5. The extreme similarity of gamma-ray spectra of several irradiated soil samples made it impractical to identify the materials by this means, at least on the basis of the spectra obtained from isotopes longer lived than 15-hr sodium-24. Apparently gamma-ray spectra are not always a "fingerprint" of the neutron irradiated sample and thus must be used with caution to trace samples to their origin.

EPIDEMIOLOGICAL STUDIES IN HUMAN POPULATIONS

EPIDEMIOLOGICAL STUDIES IN HUMAN POPULATIONS—COLORADO BIRTH AND DEATH RATE STUDY

Douglas Grahn and Harry Auerbach

PURPOSE AND METHODS

This study was designed with two main purposes. The first was to explore the possible relationships between neonatal death and the presence of uranium ore reserves, the latter taken as an indication of a higher than normal environmental radiation level. The second purpose was a much broader one but potentially with more valuable results. In the development of methods of radiation hazard evaluation in human populations, a prime source of data is the routinely and universally registered birth and death certificates. Careful evaluation of such data for the almost two hundred million persons in the United States population or selected groups in the population may lead to statistical methods of arriving at estimates of hazard where such populations are subject to predictable or measurable radiation exposures. The development of quantitative methods of using such data would be immeasurably valuable.

The ability to analyze statistically several hundred thousand detailed records by means of high speed electronic computers may lead to the development of methods of multivariate analysis of the many variables operating in human populations and allow the partitioning of such variables in hazard evaluation.

The initial analysis of neonatal death rate and infant birth weight was based on the published vital statistics and census data of the U.S. for the years 1950 to 1957, inclusive. Data on geologic environment and uranium reserves were obtained from the Division of Raw Materials, USAEC.

The second approach to this study used punch cards, supplied by the Public Health Service, for each of the 3070 counties in the country with the basic data for the more precise computation of the various indices of individual county infant death rates. These county punch cards were for the period 1953–1957.

The last phase of the study employed extremely detailed information available directly from birth certificates and death certificates. The Colorado Department of Health provided us with duplicate IBM punch cards of all birth certificate data for all births in the state in

the years 1959–1962 and all infant death certificate data for the same period. These consisted of 4800 infant deaths and 180,000 births in this period.

PROGRESS REPORT

The initial phase of this study, based on published vital statistics and uranium ore reserves, was completed and published in December 1963. The infant death rate was found to be higher in mountain regions compared to sea-level areas. No significant correlations were found between infant death rate and terrestrial radiation. A direct correlation between the neonatal death rates and altitude as well as an inverse correlation between average birth weight and altitude was found. Neonatal death rate increases and birth weight declines steadily as altitude increases. Evaluation of available experimental and clinical experience suggested that the underlying factor is the decreasing oxygen partial pressure associated with altitude increase. Hypoxia acts to inhibit fetal growth late in gestation and in turn increases the probability of neonatal death.

Analysis of the county punch card data for the state of Colorado was carried out since this state possesses populations residing in a wide range of altitudes (3000–11,000 ft.). The initial study findings of increasing neonatal death rate with increasing altitude of maternal residence were corroborated.

The 180,000 IBM cards containing the birth certificate data for the state of Colorado for the years 1959–1962 were programmed on the IBM 704 computer for analysis of the relationship of average birth weights to the following interacting variables: altitude of maternal residence, sex of infant, maternal age, and length of pregnancy. The computations and tabulations of these interacting variables are completed and multivariate analysis of the quantitative effects of these variables is now in progress.

Similarly, the 5000 death certificate cards for infant deaths in the period 1959–1962 were tabulated and computations of the various indices of infant mortality by altitude of maternal residence are now in progress.

Acute and Chronic Lethal Effects

MEASURABLE INDICATORS OF PERMEABILITY CHANGE IN RESPONSE TO Co^{60} γ IRRADIATION

Emily J. B. Christian and S. Phyllis Stearner

PURPOSE AND METHODS

In the 3-day chick, Co^{60} γ irradiation produces an early mortality, death within 24 hours. This initial response, which occurs within a few hours after exposure to a lethal radiation dose, is characterized by sluggish circulation, hypotension, edema, and hemorrhage. Similar effects have been observed in irradiated chick embryos and adult chickens.^(1, 2) In an effort to determine mechanisms involved in these permeability changes and circulatory insufficiency after irradiation, a study of the circulating plasma protein concentration and composition was undertaken. In a previous experiment, it was found that lethal doses produced an almost immediate decrease in plasma protein concentration. Thus, the alterations in the concentration of plasma protein and its principal components can be used as indicators of permeability changes that occur after sublethal and lethal exposures.⁽³⁾

Total body γ irradiation from a 5000-curie Co^{60} source was given to 3-day-old chicks in 10-minute or 24-hour exposures. Doses of 400, 700 or 1400 R were given in 10 minutes while exposures protracted over a 24-hour period consisted of 3000, 6000 or 12,800 R. In some instances, protracted exposures were interrupted in order to remove animals that were to be tested during the course of the irradiation. Blood samples for plasma protein determination were taken from both control and irradiated chicks by a standard procedure.⁽³⁾ A total of 530 irradiated chicks and 168 controls were sampled. Total plasma protein concentration was measured by the method of Lowry *et al.*⁽⁴⁾ The protein components of the plasma were separated by the Spinco paper strip electrophoresis method as described in the Spinco Instruction Manual.⁽⁵⁾ I^{131} -labeled serum albumin was used for plasma volume determinations. Chicks were anesthetized with 0.03 mg/g Nembutal. The left femoral artery was cannulated and a weighed amount of I^{131} -

labeled serum albumin consisting of about 0.1 cc of a 20 μc per ml solution was injected. A mixing time of 5 minutes was allowed before a heart blood sample was withdrawn. Determinations of plasma volume were carried out on 54 treated and 10 control chicks.

RESULTS

Short Exposures (10 Minutes)

In the chick, the early mortality that follows total-body irradiation is largely dependent on exposure time. Thus, moderate doses of ionizing radiation delivered over a short time period (e.g., 10 minutes) may result in death within 24 hours. Exposure to such a dose was quickly followed by increase in the red cell hematocrit. The normal hematocrit value was about 26%. After a lethal dose of 1400 R (more than 99% mortality within 24 hours), the hematocrit rose rapidly to 37% in less than 2 hours. The response of the sublethally irradiated group (400 R) cannot be said to vary from the normal, but there was a slight slow rise in the first 8 hours after a minimum lethal dose of 700 R. In 24 hours, this group returned to near normal levels. These effects are shown in Figure 55. If there is no change in red cell volume, then an increase in the red cell hematocrit indicates a plasma volume loss. To determine whether such a change could be demonstrated, plasma volume was determined after exposure to 700 or 1400 R. At the desired sampling time, a weighed amount of I^{131} -labeled serum albumin was injected through the cannulated femoral artery; controls were identically treated. In animals sampled within the first hour after exposure to 1400 R, apparently there was no change in plasma volume. Thereafter, the circulating volume decreased with increase in time after irradiation (Figure 56). Radiation mortality reached almost 70% in 4 hours, and sampling was discontinued. Because this method for determination of plasma volume resulted in a certain amount of

surgical stress, only the active individuals could be included. There was some selection after 2 hours, for many chicks were inactive, and some radiation mortality had already occurred. Chicks possessing normal or near normal values after 2 hours might well be considered 4-hour survivors. After exposure to 700 R, the plasma volume was only slightly decreased at 3.5–4 hours. During the period of 6–10 hours, there was little further change, and few values fell outside the control range.

In the 3-day chick, the plasma protein concentration was 38.2 mg/ml, of which 15.3 mg/ml was albumin and the remainder globulin. The loss of circulating albumin began almost immediately after exposure to 1400 R (Figure 57). After the minimal lethal dose (700 R), the albumin level fell gradually in the first 6 hours and had risen significantly by 24 hours. The response after ex-

posure to 400 R was slight and the concentration returned to near normal levels at 24 hours.

Total globulin can be separated into α , β , and γ fractions, but the concentrations of the α and β fractions are relatively small and variable. Therefore, only the total globulin concentration has been considered. Irradiation was followed by some decrease in concentration, but there was little correlation with dose. In all dose groups, the decrease was less marked than that of the albumin; concentration seldom fell below 60% of the control level.

FIG. 55.—Mean hematocrit (% RBC) after 10-minute exposures to 400, 700, and 1400 R of Co^{60} γ -rays.

FIG. 56.—Plasma volume (individual values) after exposure to 700 or 1400 R, delivered in 10 minutes, compared to mean control value.

FIG. 57.—Albumin concentration (mg/ml) after a 10-minute exposure to Co^{60} γ radiation. Mean control concentration 15.3 mg/ml.

Protracted Exposures (up to 24 Hours)

In contrast to the lethal effects of a fast exposure, e.g., 1400 R delivered in a few minutes, early death in the chick does not occur if this same amount of radiation is delivered over several hours. The plasma protein concentration during the course of lethal* and sublethal protracted exposures is of special interest in the general problem of the mechanism of early mortality and the exposure-time effect.

We have tested a range of protracted doses, up to an amount that results in 80% mortality at the end of the 24-hour exposure period (12,800 R). The two lower doses, 3000 and 6000 R, resulted in no early mortality. Each dose group was sampled during the course of the irradiation as well as at the end of the 24-hour exposure period in order to determine the time course of alterations in the plasma.

A pronounced change in the hematocrit was observed only in the highest exposure group—12,800 R (Figure 58). Increases were recorded at 3 and 6 hours after the beginning of the exposure (when 1600 and 3200 R had

* Lethal within the early mortality period (1–2 days after the beginning of the exposure).

been delivered). Values were no higher at the end of the 24-hour exposure period in individuals surviving at this point. In the two lower dose groups, the increase in hematocrit at 12–18 hours was comparable to that observed after exposure to 700 R in 10 minutes.

Exposure to 3000 or 6000 R over 24 hours resulted in only a small change in albumin concentration (Figure 59). During the exposure in the higher of these two dose

FIG. 58.—Hematocrit values during a 24-hour exposure to Co^{60} γ radiation.

groups—when half or three-quarters of the dose had been delivered—the albumin concentration was slightly lower than it was at the end of the exposure. In neither of these dose groups was mortality experienced during irradiation. In the highest dose group, 12,800 R delivered in 24 hours, there was 80% mortality at the end of the exposure. For many individuals in the group, then, a lethal dose was accumulated some time before the end of the exposure. The albumin concentration was unchanged at 3 hours but had decreased at 6 hours after the beginning of the exposure. No further change was shown at 24 hours (Figure 59). At this time, however, only 20% of the original group was available for sampling, and most of these would have been survivors of the early mortality period.

There were relatively small changes in the globulin concentration during the protracted exposures. In no case was the concentration decreased to less than 75% of the control.

In the highest protracted dose group, some chicks

were removed from the exposure room after only 6 hours of irradiation. At this time, the delivered dose of 3200 R was sufficient to result in some mortality within 10–18 hours. About 50% mortality occurred within 24 hours after the beginning of the exposure. The hematocrit was 40.2% at 6 hours and showed some additional increase through 14 hours; but there was a marked decrease in individuals surviving at 24 hours. The albumin concentration, which had fallen to 10.6 mg/ml at the end of the exposure, had decreased to 5.43 mg/ml at 14 hours. At 24 hours after the beginning of the exposure it had risen to 10.1 mg/ml.

CONCLUSIONS

Ionizing radiation may act as an inflammatory agent to produce vasodilation and increased permeability. It has been shown that a marked hypoproteinemia develops in the young chick within a short time after lethal irradiation. We postulated that a radiation induced increase in vascular permeability results in a

FIG. 59.—Albumin concentration (mg/ml) during a 24-hour exposure to Co^{60} γ radiation.

decreased protein concentration and a subsequent loss of circulating plasma volume. These effects may then result in the prominent circulatory failure associated with early lethality.

In the short exposure time group, decrease in plasma volume occurred after the initial decrease in albumin concentration, and this change may initiate the severe hypotension previously described in lethally irradiated chicks.⁽³⁾ It appears that the rate of protein loss and sub-

sequent decrease in plasma volume may be the factors that are critical for survival. (Compare the albumin concentration after exposure to 700 and 1400 R in Figure 57.) It is evident then, that it is not the low level of albumin concentration that is significant in the early mortality, but the rate at which it occurs. If this loss occurs at the gradual rate demonstrated in the 700 R group, it can be assumed that the animals are able to compensate for the alteration in composition of plasma. At 24 hours, the albumin concentration appears to approach normal levels.

Dependence of the permeability changes on duration of the exposure as well as on total dose is demonstrated in the protracted exposure groups. When 3000–6000 R are delivered over a 24-hour period, the effects on plasma protein concentration and hematocrit are not greater than those recorded after exposure to 700 R delivered in 10 minutes. Only in chicks exposed to 12,800 R is the magnitude of the effects equivalent to that following a 10-minute exposure to 1400 R.

STUDIES IN THE COMPARATIVE PHYSIOLOGY AND ANATOMY OF RADIOSENSITIVITY AND AGING

George A. Sacher and Everett F. Staffeldt

PURPOSE AND METHODS

The necessity to investigate the late effects of ionizing radiations in small, short-lived, experimental animals carries with it a commitment to discover the factors that govern longevity and aging in mammals. If two or more factors are involved, and if the factors differ markedly in radiosensitivity, no single scaling factor will be sufficient to estimate human life shortening on the basis of animal data.

PROGRESS REPORT

Concern about this problem led us to set up a study of the allometry of length of life in mammalian species. This work has been done at intervals over the past five years. Thus far the study has produced strong evidence that mammalian longevity is governed almost entirely by two factors. The first factor is related to over-all energy expenditure. Species body weight or metabolic rate is a good measure of the energy dissipation factor, and weights of certain visceral organs, such as heart or liver, are also fair estimators of it. The second factor has to do with the goodness of organization and control of physiological performance. The best quantitative measure of this organizational factor that has been found thus far is brain weight. The following rule can be stated: If two species have equal adult body weights,

Direct measurements of plasma volumes in the protracted exposure groups will be undertaken and may provide more conclusive data. It is in the highest protracted exposure group that the greatest increases in hematocrit values were recorded, suggesting that a severe decrease in plasma volume has occurred.

REFERENCES

1. Stearner, S. P., M. H. Sanderson, E. J. Christian and S. A. Tyler. Modes of radiation death in the chick embryo. 1. Acute syndromes. *Radiation Res.* 12, 285–300 (1960).
2. Stearner, S. P., M. Sanderson, E. J. Christian and A. M. Brues. Initial radiation syndrome in the adult chicken. *Am. J. Physiol.* 184, 134–140 (1956).
3. Christian, E. J. B. and S. P. Stearner. Early alterations in plasma proteins in gamma-irradiated chicks. Argonne National Laboratory Biological and Medical Research Division Semi-annual Report, January through June, 1962. ANL-6723, pp. 5–9.
4. Lowry, O. J., N. J. Rosebrough, A. L. Farr and R. J. Randall. Protein measurements with folin phenol reagents. *J. Biol. Chem.* 193, 265–275 (1951).
5. *Spinco Model R Electrophoresis System Instruction Manual*. Beckman/Spinco Division, Palo Alto, California.

but differ in adult brain weight, the species possessing the larger brain will have the greater longevity. Among species for which accurate longevity data are available no exception to this rule has yet been found. An estimation formula using the two factors estimates human longevity accurately. If body weight alone is used to estimate longevity, the predicted longevity for man is 25 years, which is in error by more than a factor of three.

The implication is inescapable that a complete program of investigation of the toxic actions of ionizing radiations must evaluate the influence of body size and brain size separately. We are now preparing to take up this problem in experimental terms. The approach will be to assemble a set of rodent species that covers an appreciable range of these two dimensions.

We will certainly use *Mus musculus* (the wild ancestor of the laboratory mouse) and *Peromyscus leucopus* (the deer mouse of our fields). *Peromyscus* has a brain weighing 600 mg as against the 400 mg brain of *Mus*, and *Peromyscus* lives 5+ years as against the 3+ of the laboratory mouse.

We began a *Peromyscus* colony in the summer of 1963 and it has been very successful. *Peromyscus* adapts well to the laboratory and is a prolific breeder. The infantile mortality rate is low and there have been virtually no

deaths from disease in our adult herd. The definitive experiment, when it begins, will include the following studies on both species:

1. Acute radiation responses.
2. Life table and pathology.
3. Longitudinal study of "good" age-dependent physiological variables, including metabolism, body composition and stress responses.
4. Measurement of goodness of physiological regulation.
5. Measurement of psychological capabilities, such as discrimination, learning, memory.
6. Measurement of radiation life shortening.

Additional species now being evaluated as possible test species include:

1. *Tamias striatus* (chipmunk), a long-lived and large-brained member of the squirrel family. We have no hopes of breeding this species, but they seem to do well in captivity. It will be necessary to provide more adequate caging for chipmunks if we decide to use them.

2. *Sigmodon hispidus* (cotton rat) is a native cricetid rodent. They are fairly prolific breeders, but the mortality rates in the young are excessive. We should be able to maintain them with some improvement in the breeding arrangements.

3. *Neotoma sp.* (wood rat and pack rat) is a lively, intelligent cricetid. They reproduce in the laboratory but litter sizes are small.

4. *Mastomys (Rattus) natalensis* (the multimammate mouse) is a small murid which is being brought into laboratories because it has a better karyotype than most mammals. It has 36 chromosomes that are more varied in length and shape than mouse or rat chromosomes.

Mastomys is a good breeder in the laboratory, so we expect to make it a test species for the comparative program and also a test object for a contemplated large-scale study of the effects of small radiation exposures, in which we will measure the dose-response curves for cytogenetic damage and for induction of specific neoplasms.

5. *Microtus sp.*, the meadow vole, has not yet been successfully transplanted to the laboratory, but this is attributable to a few major mistakes which can be corrected in the future. It is not likely that we will decide to use this species, however.

6. *Dipodomys sp.*, the kangaroo rat, was purchased in 1963 but the surviving animals were killed early in 1964. We cannot offer it a good environment at a reasonable cost.

7. *Cricetus auratus*, the hamster, will almost certainly be employed. Our breeding colony has not been very productive in terms of number of litters or survival of newborn. Some small improvements in the breeding arrangements are indicated.

CONCLUSIONS

The ultimate intention of this experimental program is to have four to six species established as test material, and to carry out a battery of measurements of the kind described above for *Mus* and *Peromyscus*. It is believed that the information so obtained will increase the capability for interspecies prediction of lifetime radiation effects. It is also believed that the study of aging in species or subspecies differing in brain size will add a new dimension to the biology of aging.

EFFECT OF LIFETIME PERIODIC GAMMA-RAY EXPOSURE ON THE SURVIVAL AND PATHOLOGY OF GUINEA PIGS

George A. Sacher, Douglas Grahn, and Robert J. Robertson

PURPOSE AND METHODS

The purpose is to measure the life shortening in guinea pigs given small periodic gamma-ray exposures for the duration of life. The studies by Lorenz *et al.* established the high sensitivity of these animals to daily doses in the range of 4.4 to 8.8 R/day, but his samples were too small to permit firm conclusions about the sensitivity at daily dosages of 1 R/day and below.

A total of 348 guinea pigs was entered into the periodic exposure condition over a one-year period and another 48 two years later. Weekly dosages ranged from 1.35 to 36 R. Body weights were taken throughout the experiment. Tissues were taken from over 90% of the animals for histopathological examination.

PROGRESS REPORT

This project is now in its seventh year, and the dying out of the initial group is practically complete. An interim report on survival appeared earlier.⁽¹⁾ At 1.3 R/week, given in a single 12-hour fraction weekly, no difference between control and irradiated groups is detectable either in the survival or the pathology (120 controls and 90 exposed). At 5.2 R/week (given in four 12-hour fractions) survival of the exposed group (48 pigs) exceeds control for four years, but in the succeeding two years (accumulated doses greater than about 1100 R) the exposed group died out rapidly. At 13 R/week and above, survival of exposed groups is less than control and decreases with increasing weekly dose.

Histopathological study of the tissues is being carried out by Dr. John Rust, University of Chicago, and will soon be complete. Several tumors are present, including lymphatic leukemia, ovarian tumor, and lung tumor. Kidney lesions and amyloidosis are also observed frequently in these animals. The lymphatic leukemia seems to be increased by radiation exposure.

In the experiment described, pigs were housed two per cage. In a subsequent experiment, 24 controls and 24 pigs given 5.2 R/week were housed one per cage. After 1250 days, there is no difference between the two groups in survival (15 and 16 survivors for irradiated and control, respectively), but survival is poorer than in the initial experiment.

CONCLUSIONS

We now have better information about the life-

shortening effects of small periodic exposures in guinea pigs, and it is clear that the high sensitivity observed in the 4.4–8.8 R/day range is not present when exposure averages 1 R/day or less.

It is evident that we do not yet know the environmental factors that govern susceptibility to infectious and degenerative diseases in laboratory populations. The realization of this fact has motivated us to give much attention to the deliberate control of the physical environment in the Janus toxicity program.

REFERENCE

1. Sacher, G. A., D. Grahn and R. J. Robertson. Survival of guinea pigs given low daily gamma-ray exposure—fourth year. Argonne National Laboratory Biological and Medical Research Division Semiannual Report, January through June, 1962. ANL-6723, pp. 10–15.

STANDARDIZATION OF A SMALL FISH AS A TEST OBJECT FOR RADIOBIOLOGICAL AND AGING STUDIES

George A. Sacher and Emil G. Johnson

PURPOSE AND METHODS

There is need for a small poikilothermic water-living vertebrate to be used in the Janus toxicity program in studies of the influence of physical factors such as temperature, oxygen tension, nitrogen or rare gas pressure, pH, etc., on the acute and chronic effects of gamma rays and fast neutrons. It is desirable that this organism have a high fertility, short maturation time, and short life-span.

PROGRESS REPORT

The first species tried was the guppy (*Lebistes reticulatus*), which is small, hardy, and easily procured. A fairly thorough study was made of the guppy's acute and chronic radiation responses, as will be described below, but the species was not entirely satisfactory because of its slow maturation (6–7 months) and comparatively long life-span (in excess of 2 years).

Early in 1964 we obtained from Dr. Roy Walford of U.C.L.A. a batch of eggs of a small South American fish, *Cynolebias adloffi*. These are so-called "annual fish" because they live in lakes and rivers that dry up in summer. The fish lay eggs that lie dormant during the dry season and hatch when the rains come (we had complete hatching out within one hour of putting the eggs in water). Growth is rapid (2 or 3 months to sexual maturity) and maximum longevity is established by Dr. Walford to be less than 18 months. In spite of the short life-span, the young fish are hardy.

The eggs were cultured in the Animal Quarters and we had a 90+% hatch from 50 eggs with very little mortality in the subsequent 5 months. We have not yet collected eggs for continued breeding. When breeding is successful, further steps will be taken to develop this fish as a radiobiological test object.

Although the guppy project has been terminated, it yielded excellent data. Survival times under exposure to X-rays given in 3 treatments per week for the duration of life have been measured for weekly doses ranging from 75 R to 3000 R. The relation of life shortening to daily dose is of the same form as for the mouse, but the guppy has a life shortening coefficient about one-fourth that of the mouse. The dose-survival curve has been measured for single doses and for doses divided into 3 or 5 fractions. These data yield an estimate of the recovery constant of the guppy and its dependence on size of fraction.

CONCLUSIONS

When breeding and rearing of *Cynolebias* is standardized, the fish should have many uses. The eggs are sturdy and can be manipulated in many ways. Whole-body irradiations can be done under a wide range of environmental conditions. The tail provides a favorable geometry for study of the effects of particle beams on cells *in vivo*. The short life-span makes feasible a thorough study of radiation effects on longevity.

EFFECTS OF IONIZING RADIATIONS ON SURVIVAL IN THE CHICKEN

S. Phyllis Stearner and Emily J. B. Christian

PURPOSE AND METHODS

White leghorn roosters, irradiated at 3-4 days after hatching, are being maintained for duration of life to determine long-term effects on survival time. No long-term observation of chickens maintained under laboratory conditions is known; therefore, observations of control birds form a significant part of the study. The radiation exposures were either to 200 kV X rays or to Co^{60} γ rays. All X-ray exposures were delivered in 30 min; γ ray exposures were given in 6-60 min or in 24 hours in order to investigate the effect of exposure time on dose effectiveness. Doses covered the 30-day lethal range. Survivors at 30 days were caged individually and observed regularly for duration of life.

PROGRESS REPORT

Survival of the entire control group has been tabu-

lated only through 4 years of age. Mortality during the first year was high (about 15%), but thereafter the survival curve seemed to approximate an arithmetic straight line. On the basis of mortality in the older birds of the group, there appears to be an increase in the number of deaths between 6 and 7 years. X-irradiated roosters (dose of 1000-1200 R) showed nearly 50% mortality at the end of the first year (based on those alive at 30 days). Between 1 and 5 years, the survival curve paralleled that of the control, and, as indicated in the control group, mortality was high between 6 and 7 years. Survival information in the γ -irradiated groups is not yet sufficiently far advanced to permit any specific comments. Generally, however, mortality appears to be dependent on exposure time as well as on total dose.

INFLUENCE OF IONIZING RADIATIONS ON GROWTH IN THE CHICKEN

S. Phyllis Stearner and Sylvanus A. Tyler

PURPOSE AND METHODS

The growth curve of the chicken consists of two phases. There is an initial rapid growth phase that occurs over a period of about 300 days after hatching. An extended period of slow sustained weight increase follows, continuing for several years. We are attempting to fit the rapid growth phase to the Gompertz function

$$W = W_0 e^{(A/a)(1 - e^{-at})},$$

where W is the weight at time t , W_0 is the starting weight, A and a are constants.

PROGRESS REPORT

Three-day-old, male, white leghorn chicks were ex-

posed to doses of Co^{60} γ rays in the 30-day lethal range. Exposures were delivered over periods of 6-60 min or 24 hours. In the analysis now in progress, values for the constants in the growth function will be determined in individual animals and control and irradiated groups compared. Of special interest is a comparison of the influence of dose and of exposure time in the radiation effect on growth.

In irradiated birds, weight increase after 300 days is appreciably less than in controls or is absent. The amount of deviation from the control increase appears to be positively correlated with dose.

IRRADIATION OF MOUSE BONE MARROW CELLS WITH FISSION NEUTRONS AND Co^{60} γ RAYS

Howard H. Vogel, Jr.

PURPOSE AND METHODS

The purpose of this project was to study the comparative effects of fission neutrons and Co^{60} γ rays on the bone marrow cells of the mouse. Till and McCulloch⁽¹⁾ have described a direct measurement of sensitivity of marrow cells exposed *in vitro* to X or γ rays. Their method involved the intravenous injection of marrow

cells into isologous hosts previously exposed to supralethal total-body irradiation. Colonies of proliferating cells appeared in the spleen as gross nodules that could be counted 10 or 11 days after injection. This spleen-colony method has been utilized to compare the effects of fission neutrons and Co^{60} γ rays on mammalian marrow cells.

Bone marrow cells were obtained from the femurs of young adult CF No. 1 female mice by flushing cells from the marrow cavity with a small volume of Tyrode's solution. Nucleated cells were counted in a hemocytometer, and appropriate dilutions were made in Tyrode's solution so that a known number of cells could be injected intravenously into host mice. Each mouse received 0.1 cc via caudal vein.

Preliminary experiments with unirradiated marrow cells showed that the number of colonies observed in the spleen 10 days after such injections was approximately linearly related to the number of nucleated marrow cells injected. This relation held true within certain limits: at least 10^4 nucleated marrow cells were necessary before one could detect visible colonies in the spleen of the host mouse. When more than 10^5 cells were injected, resulting splenic colonies became so numerous and overlapping that counts became difficult and inaccurate.

FIG. 60.—Dose-survival curve for mouse bone marrow cells exposed to single doses of gamma rays. The surviving fraction of the nucleated marrow cells was based on the number of colonies observed in the spleens 10 days after injection.

PROGRESS REPORT

Dose-Survival of Mouse Bone Marrow Cells

Co^{60} γ Rays.—Figure 60 illustrates the survival of marrow cells, as measured by this spleen-colony method, after single exposures to γ rays. The cells were exposed in the gamma-neutron radiation chamber.⁽²⁾ They were irradiated *in vitro* in 2–3 cc Tyrode's solution in small test tubes of cellulose nitrate. The dose rate was

6–7 rads/min. The marrow cells were kept cold until injection into the host mice. Host mice were exposed to 900–1000 rads of γ rays in a single whole-body irradiation. This dose was found necessary to prevent significant endogenous colony formation from the host's own marrow cells. Our data indicate that following such an exposure less than one colony will be found in the spleens of 10-day survivors. Marrow cells were injected into irradiated hosts within a few hours after irradiation.

FIG. 61.—Dose-survival curve for mouse bone marrow cells exposed to single doses of fission neutrons.

There appears to be a definite shoulder to the curve illustrated in Figure 60. The D_0 for these mammalian cells exposed to γ rays was found to be approximately 120 rads. These data confirm the work reported by McCulloch and Till on the effects of X and γ rays on bone marrow cells in several mouse strains.⁽³⁾

Fission Neutrons.—In Figure 61 a similar dose-survival curve is illustrated for exposure of murine marrow cells to fission neutrons at Argonne's CP-5 research reactor. All neutron exposures were carried out at a dose rate of 8 to 10 rads/min with the reactor operating at 4.0 to 4.5 MW. The experimental procedure was the same as that previously described for γ irradiation, except that the host mice were exposed to at least 400 rads of fission neutrons instead of 900 to 1000 rads of γ rays. In this case there appears to be no shoulder to the dose-survival curve, the exponential portion extending straight to the 100% survival point. The D_0 for neutron irradiation appears to be approximately 50 rads.

Fission neutrons thus appear to be approximately 2.2 times as effective as Co^{60} γ rays for this biological endpoint involving marrow cells irradiated *in vitro* but proliferating *in vivo*.

Additivity Experiments Using Both Radiations

Figure 62 summarizes six "additivity" experiments in which marrow cells were exposed to both radiations simultaneously. These irradiations were brief, from 5 to 8 minutes. The two solid lines represent the dose-survival curves for fission neutrons and Co^{60} γ rays

FIG. 62.—Additivity experiments: Survival of mouse bone marrow cells exposed to mixed doses of gamma rays and fission neutrons administered simultaneously *in vitro*. (Triangles represent separate additivity experiments.)

given as single exposures. The six points represented by triangles illustrate individual experiments in which mouse marrow cells were exposed to both radiations concurrently. In these mixed exposures the neutrons contributed approximately 75% of the biologically effective dose (calculated in rems) if a figure of 2.2 is used for the relative biological effectiveness of neutrons to gamma rays. The six experimental points from these simultaneous exposures to the two radiations all lie very close to the γ ray dose-survival data and within the experimental error of the γ ray curve.

It can be concluded therefore that no significant departure from additivity was found when the two radiations were tested on the mouse hematopoietic system as measured by the spleen colony method. In previous experiments, involving spleen weights several days

after exposure to mixtures of these two radiations, we were also unable to demonstrate a significant departure from additivity.⁽⁴⁾

Early Recovery or Repair of Hematopoietic Cells Measured by Fractionation Experiments

Several exposures to paired doses of the two radiations are illustrated in Table 10. In the case of the neutron exposures, the surviving fraction of the injected marrow cells, as measured by colony forming units in the spleen, was no higher after paired doses than after single exposures. There was, therefore, no evidence of early recovery in the four-hour period between two neutron exposures. In contrast, similar paired doses of γ rays showed marked recovery within a few hours of exposure. Paired doses of γ rays, similar to the single illustrated example, indicate that recovery does begin within 1 or 2 hours after exposure and reaches a maximum by 5 hours. When two exposures to 400 R of γ rays are separated by such an interval, colonies in the spleen may be 4 or 5 times as numerous as those found after a single exposure of marrow cells to the same total dose (800 R) of γ rays.

TABLE 10
RECOVERY EXPERIMENTS WITH
MOUSE BONE MARROW CELLS

	DOSE, RADS	SURVIVING FRACTION, %
Co^{60} γ rays	250	7
	125←4 hours→125	28
Fission neutrons	40	41
	20←4 hours→20	35
	80	20
	40←4 hours→40	15
	100	15
	50←4 hours→50	11

Using this method of studying early repair in mouse hematopoietic cells, such paired dose exposures have been carried out with intervals varying from 1 to 24 hours with each radiation. Marrow cells have been irradiated with fission neutrons for comparisons between *in vivo* and *in vitro* exposures.

Fractionated exposures have indicated a marked difference in early repair following exposures of marrow cells to fission neutrons and Co^{60} γ rays.

Spontaneous or Endogenous Colony Formation

Comparative studies of the "spontaneous" colonies formed in the spleens of mice exposed to varying sublethal doses of the two radiations without any bone marrow cells being injected have been carried out. These experiments strongly suggest that even after a supralethal

dose of fission neutrons (400 rads), some blood-forming cells of the marrow escape the effects of the dense ionizing columns of protons resulting from the neutron irradiation. Some colonies are usually found in the spleens of mice exposed to 375–400 rads of fission neutrons (LD 90–99/30), whereas 900 rads of Co^{60} γ rays (LD 50–60/30) reduces the average number of colonies to less than 1 per spleen. It appears from these data that more of the colony-forming cells in the marrow are spared after high doses of neutrons than after similar doses of gamma rays. We believe this is a result of the more uniform distribution of ion pairs resulting from the gamma rays. The spleen colony method appears to be useful in correlating the physical distribution of external radiations with their biological effects.

This experimental method should be important in

correlating damage to mammalian blood-forming cells with acute lethality following these two radiations. The establishment of the relations of cellular damage to radiation mortality is one of the objectives of the Janus program.

REFERENCES

1. Till, J. E. and E. A. McCulloch. A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. *Radiation Res.* 14, 213–222 (1961).
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).
3. McCulloch, E. A. and J. E. Till. The sensitivity of cells from normal mouse bone marrow to gamma radiation *in vitro* and *in vivo*. *Radiation Res.* 16, 822–832 (1962).
4. Jordan, D. L., J. W. Clark and H. H. Vogel, Jr. The additivity of gamma rays and fission neutrons in producing spleen weight reduction. *Radiation Res.* 4, 77–85 (1956).

Cellular and Physiological Effects

THE INTESTINAL FLORA OF THE BCF_1 MOUSE

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

PURPOSE AND METHODS

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

1) To establish the kinds of bacteria, their distribution and the numbers of organisms in the various regions of the gut.

2) To determine the influence of such factors as antibiotics, starvation, diet and irradiation on the distribution of the flora.

At sacrifice, 1-cm lengths of gut were isolated and removed from the duodenum, the mid-zone of the small intestine, a section 3 cm from the ileocecal junction, the terminal centimeter of the ileum, and from the ascending colon. The segments of gut were opened, and both the contents and the gut were put into 1 ml of sterile saline and serial dilutions made. Following culture both aerobic and anaerobic organisms were identified by morphology, staining and appropriate biochemical tests. Estimates of the numbers of bacteria were made from growth of colonies from serial dilutions.

PROGRESS REPORT

It will be seen in Table 11 that the flora have been classified into five groups. The important but little understood group of organisms known as bacteroides

have not been included because of the inherent difficulties in culturing them. Their presence in one of the experimental groups is discussed later. The number of general groups of bacteria found on culture was relatively small. There was considerable variation in the number of bacteria in the various regions of the different animals. Although not sterile, the duodenum contained fewer organisms, both in numbers and species.

It was found that there was a close correlation between the amount of contents in the lumen and the number of bacteria. When the mice were maintained on glucose and water, the number of bacteria in the small intestine was reduced. These results are consistent with the observations of Dixon⁽¹⁾ on the importance of

TABLE 11
NUMBERS OF ORGANISMS IN VARIOUS REGIONS OF INTESTINAL TRACT OF BCF_1 MICE GIVEN AS RANGES OF NUMBERS OF BACTERIA IN 1-CM LENGTHS

Region	Micro-cocci	Entero-cocci	Yeasts	Coli-forms	Lacto-bacilli
Duodenum.....	0	0–10 ²	0–10 ³	0–10 ²	10–10 ⁴
Small intestine mid-zone.....	0	0	0–10 ⁴	0	0–10 ⁵
Ileum 1.....	0–10 ⁵	0–10 ⁴	0–10 ⁵	0–10 ⁵	10 ² –10 ³
Ileum 2.....	0–10 ⁵	0–10 ⁴	0–10 ⁵	0–10 ²	0–10 ⁵
Colon.....	0–10 ⁵	0–10 ⁶	0–10 ⁷	0–10 ⁷	10 ⁵ –10 ¹⁰

peristalsis in flushing the flora to the distal regions of the gut.

Neomycin treatment for five days resulted in a relatively greater depression of the flora of the colon and the terminal ileum than in the upper regions of the small intestine. Bacteroides were isolated from all regions; presumably the depression of some of the other groups of bacteria provided a more suitable environment for the growth of the bacteroides.

THE EFFECT OF A DIET CONTAINING SODIUM DEOXYCHOLATE ON THE INTESTINAL MUCOSA OF THE MOUSE

R. J. M. Fry and Everett F. Staffeldt

PURPOSE AND METHODS

Among the factors that may influence the life-span of the cell's motility, intestinal flora and bile acids are of particular interest because of their interplay. If intestinal motility is reduced a change in the distribution of flora occurs with an increase in the bacterial population of the upper intestine. The conjugated bile acids are split by microbial action with the production of cytotoxic substances such as deoxycholic and lithocholic acids. In a number of clinical conditions and following whole-body irradiation some of the changes in structure and function of the intestine may result from alterations of the normal interplay of these three factors.

This report is concerned with part of our investigations into the question of whether bile and bile acid metabolites influence the life-span of the intestinal cells. While it has been shown that *in vitro* incubation with deoxycholic acid caused marked damage to the villi⁽¹⁾ and Sullivan demonstrated the importance of the bile acids in postirradiation diarrhea⁽²⁾ there is little information on the *in vivo* effects of varying the quantity or type of bile acid in the diet.

RESULTS

BCF₁ mice, about 100 days of age, were maintained on a) a synthetic diet (casein 18%, corn oil 10%, sucrose 68%, salt mixture No. 2 USP 4%, complete vitamin fortification mixture from Nutritional Biochemicals Corporation), b) the same diet with 2% sodium deoxycholate added. Mice were kept under observation for over one month, and sacrifices were made at 2, 8, and 39 days after starting the diet. The mice fed the control

So far only two mice have been investigated following irradiation, but the findings were considered clear-cut. Three days following 1000 R whole-body gamma irradiation there was an increase in the number of organisms in the upper intestine and *E. coli*, Micrococci and *Clostridium perfringens* were found in the duodenum.

REFERENCE

1. Dixon, J. M. S. The fate of bacteria in the small intestine. J. Pathol. Bacteriol. 79, 131-140 (1960).

synthetic diet showed no gross or microscopic changes. Mice in the experimental group appeared sick within 2 days; they were listless, and their fur was ruffled. Two days later the mice were active and appeared normal except their fur was now oily. This change in the appearance of the fur gradually disappeared in about 3 weeks.

At sacrifice on day 2 the upper intestine was more translucent and thinner than normal. The gall bladder was distended and at least three times the normal size. At 8 days the gross appearance of the lower intestine was similar to that of the upper intestine at the early sacrifice interval. There was a striking hyperemia of the entire length of the small intestine, but otherwise the appearance of the upper intestine was almost normal. The gall bladder was still markedly enlarged. At 39 days the intestine appeared normal, the gall bladder had decreased in size, but the wall was thickened.

The microscopic appearance of the duodenal villi at day 2 was abnormal. There was separation of the mucosal cells from the underlying structures with edema and proteinaceous material in the lamina propria. In the ileum at 8 days there were partial denudation and a decrease in the number of goblet cells (Figure 63). At later sacrifice intervals the appearance of the villi was almost normal, and there was evidence of increased proliferation in the crypts. Perhaps the most interesting result was the marked increase in proliferation of the tissues in the wall of the gall bladder. Subsequent experiments have shown that the initial labeling index (the % of cells labeled 1 hour after the injection of tritiated thymidine) increases from virtually zero to about 15% within four days on the diet.

CONCLUSIONS

The results demonstrate the marked *in vivo* cytotoxicity of sodium deoxycholate and the well-known powers of repair by the intestinal mucosa.

The increase in the size of the gall bladder is thought to be due to the choleretic effect of the bile acid. The cause of the increased proliferation of all the tissue elements is not clear. Jacoby⁽³⁾ found similar proliferation in the gall bladder after tying the common bile duct.

Since the mucosal cells can be stimulated to prolifer-

ate, this tissue will be a useful test system and has the advantage that repeated stimuli may be employed.

REFERENCES

1. Dawson, A. M. and K. J. Isselbacher. Studies on lipid metabolism in the small intestine with observations on the role of bile salts. *J. Clin. Invest.* **39**, 730-740 (1960).
2. Sullivan, M. Dependence of radiation diarrhea on the presence of bile in the intestine. *Nature* **195**, 1217-1218 (1962).
3. Jacoby, F. Mitotic activity in the gall bladder epithelium of the guinea pig after ligation of the common bile duct. *J. Physiol.* **119**, 21p (1953).

FIG. 63.—Ileum of mouse on diet containing 2% sodium deoxycholate for eight days

FURTHER STUDIES ON THE LOCALIZATION OF BRAIN INHIBITOR IN CHICK DEVELOPMENT

Arthur K. Katoh

PURPOSE AND METHODS

Since the theory of specific inhibition in differentiation was elaborated by Rose in 1952,⁽¹⁾ several workers have used the embryonic chick neural system in testing the hypothesis. Lenicque⁽²⁾ and Braverman⁽³⁾ showed that adult brain extracts injected into 1-day old fertile eggs resulted in homologous brain defects in the developing embryo. Using an *in vitro* system, Katoh⁽⁴⁾ also demonstrated the inhibitory activity of adult brain extracts on the neural system of developing embryos. Also, the inhibitory activity has been shown to reside in the soluble protein fraction of the brain extracts.⁽⁵⁾ Inasmuch as the specific inhibition theory needs further experimental verification and characterization of the specific inhibitors, as pointed out by Barth in her review,⁽⁶⁾ we undertook to label the soluble protein fraction and using the *in vitro* system, to follow the incorporation of the labeled extract into the embryo by autoradiographic methods. It was the purpose of this investigation to determine whether any specific incorporation of labeled brain extracts would occur in embryos manifesting specific neural defects.

Fertile white leghorn eggs were obtained weekly from Creighton Brothers, Warsaw, Indiana. The method of culturing chick embryos has been previously reported.⁽⁴⁾ Brain extracts were prepared from chicks 10–15 days of age according to the procedure reported⁽⁵⁾ with the following additional modifications. The final supernatant fraction, after centrifugation in the Spinco L preparative ultracentrifuge at $100,000 \times g$ for 60 minutes, was precipitated at saturation with ammonium sulfate. The precipitate was collected by centrifugation, dissolved in phosphate-buffered chick Ringer's solution, pH 7.6, and dialyzed against the chick Ringer's solution overnight. Samples were then taken for protein and radioactivity measurements by the Folin procedure and in the Packard Tri-Carb liquid scintillation spectrometer.

Chicks obtained from Kerr Chickeries, Frenchtown, New Jersey, were used. One hundred microcuries of DL-leucine-4,5- H^3 were injected into the wing vein of each chick used. Chicks were sacrificed 6 hours after injection, and the brains were removed, homogenized, and centrifuged as described.

Early chick embryos, stages 4 and 5 of Hamburger and Hamilton,⁽⁷⁾ were cultured on media of the following composition: 1% Bacto-agar in chick Ringer, 2 parts; albumen extract, 1 part; brain extract, 1 part. The brain

extract used had a specific activity of 5730 counts/minute/mg protein.

Embryos were cultured for approximately 18 hours, fixed in Carnoy's fluid, dehydrated, paraffin embedded, sectioned at 5 microns, and prepared for autoradiography, by dipping into Kodak NTB-3 emulsion. Thereafter, the slides were stored in black, light-tight boxes and developed in D-19 developer after 7 days in the refrigerator.

PROGRESS REPORT

The results obtained here have confirmed the previously reported cases of inhibitions, insofar as the gross, qualitative picture is concerned. Of the 18 embryos cultured on brain extract medium which were at either stages 4 or 5, 15 showed specific neural abnormalities. The remaining 3 cases failed to develop. Prominent effects noted were the failure of the closure of the neural plate anteriorly, the complete absence of optic vesicles, and the absence of neuromeric differentiations in the anterior brain region. The posterior spinal cord region, in all brain inhibited embryos, was normal. Control embryos were explanted onto normal media which consisted of equal parts of albumen extract and agar. All 11 explants developed normally, and showed no brain abnormalities. An important confirmatory feature of this experiment lies in the failure of the embryos beyond stage 5 to be affected by the brain extracts. This has already been reported and emphasized in the previous report.⁽⁴⁾ None of the 13 embryos beyond stage 5 explanted onto brain extract media showed brain defects, and all were normal. The data thus far add to the accumulating evidence in the literature supporting the concept of specific inhibition in differentiation.^(8–13) However, examination of the autoradiographs, particularly in the most severely affected embryos, indicated a failure of any specific incorporation. The isotopic label was present in all cases in general and widespread distribution throughout the longitudinally sectioned embryos, and no specificity of incorporation was observed. Inasmuch as this negative aspect of the investigation is a crucial disappointment, it nonetheless is not a deterrent to further investigations into the nature of specific inhibitors. It has served to emphasize that the fractionation of the extracts thus far achieved is, as yet, still in a crude stage. Further refinements in the isolation procedures are in order, and once achieved, might len

themselves to another attempt at specific incorporation studies in the embryo.

REFERENCES

1. Rose, S. M. A hierarchy of self-limiting reactions as the basis of differentiation and growth control. *Am. Naturalist* **86**, 337-354 (1952).
2. Lenicque, P. Studies on homologous inhibition in the chick embryo. *Acta Zool.* **40**, 141-202 (1959).
3. Braverman, M. H. Regional specificity of inhibition within the chick brain. *J. Morphol.* **108**, 263-285 (1961).
4. Katoh, A. K. Polarized inhibitory control of differentiation in the early chick embryo, studied *in vitro*. *J. Morphol.* **108**, 355-376 (1961).
5. Katoh, A. K. Localization of inhibitors of neural differentiation in chick embryos. *Nature* **192**, 997-998 (1961).
6. Barth, L. J. The developmental physiology of Amphibia. *Physiology of Amphibia*, ed. John A. Moore. Academic Press, New York, 1964, pp. 469-544.
7. Hamburger, V. and H. L. Hamilton. A series of normal stages in the development of the chick embryo. *J. Morphol.* **88**, 49-92 (1951).
8. Rose, S. M. Cellular interaction during differentiation. *Biol. Rev.* **32**, 351-382 (1957).
9. Rose, S. M. Polarized inhibitory effects during regeneration in *Tubularia*. *J. Morphol.* **100**, 187-206 (1957).
10. Tucker, M. Inhibitory control of regeneration in nemertean worms. *J. Morphol.* **105**, 569-600 (1959).
11. Clarke, R. B. and D. J. McCallion. Specific inhibition of differentiation in the frog embryo by cell-free homogenates of adult tissues. *Can. J. Zool.* **37**, 129-131 (1959).
12. Clarke, R. B. and D. J. McCallion. Specific inhibition of neural differentiation in the chick embryo. *Can. J. Zool.* **37**, 133-136 (1959).
13. Smith, S. D. Specific inhibition of regeneration in *Clymenella torquata*. *Biol. Bull.* **125**, 542-555 (1963).

STUDIES ON THE PROBLEM OF INCREASED VASCULAR PERMEABILITY IN THE CHICK

Arthur K. Katoh, Margaret H. Sanderson, and S. Phyllis Stearner

PURPOSE AND METHODS

Previous studies from this laboratory have shown that Co⁶⁰ gamma irradiation in the chick causes alterations in the plasma protein concentration.⁽¹⁾ This phenomenon has been used as an indicator of permeability changes that ensue subsequent to sublethal and lethal exposures.

The objectives of this continuing study have been to follow the development of increased capillary permeability in an effort to understand the physiological and morphological changes attendant to irradiation that result in increased vascular permeability and insufficiency.

Various experimental approaches have been tried and are in progress; they will be described in the sequence in which they have been undertaken.

PROGRESS REPORT

Cinematography

The initial approach to this general problem of altered vascular permeability lay in the use of 2-day chick embryo cultures which were cinematographically observed. The area pellucida of the chick embryo at about 40 hours' incubation age possesses many fine vascular networks. Such embryos, when stretched on a glass ring and cultured on a nutrient agar medium according to the technique of New,⁽²⁾ afford an opportunity of observing the capillaries of the embryo *in vitro*. The embryos were cultured in 35 × 10-mm plastic Petri dishes, covered with a layer of light mineral oil to prevent desiccation,⁽³⁾ and placed in the microscope chamber which was kept at 38° C. A gas mixture of 15% oxygen and 5% carbon dioxide, without which the embryos could not survive, was piped into the chamber.

Eggs of the desired incubation stages were prepared for irradiation as described in a previous report.⁽⁴⁾ After a 15-min exposure to doses that varied from 1500-1800 R, embryos were explanted onto the nutrient medium, composed of equal parts of albumen extract and 1% Bacto-agar. Embryos were photographed on 16-mm color movie film, Kodachrome II, Type A.

Our cinematographic observations on irradiated, cultured embryos have provided us with pictures of vascular channels which become occluded with blood cells with a resultant plugging of the vessels. This is recorded in pictures in which the flow of plasma and blood cells is seen as in a somewhat analogous situation when a river is obstructed by a dam. Our observations have hinted to us that these blood cells become unusually sticky and adhere to one another. Frequently, in short intervals after irradiation, we can see large aggregates of cells coursing through the vascular channels. Ultimately, these aggregates become larger and larger, until they completely block up the channel. This phenomenon occurs in a number of sites, and probably results in extensive hemorrhage seen in irradiated animals. The sinus terminalis appears to be the initial site of the formation of the hemorrhages.

Electron Microscopy

Since the resolution offered by light microscopy did not provide detailed answers to the morphological alterations concomitant with altered permeability, it was felt that electron microscopic studies would provide some answers, or if not, some interesting observations. Accordingly, a choice of tissue and radiation dose had to be determined. Since the previous studies had utilized

the 3-day chick, it was felt that continued use of this material would be advantageous. The mesentery was the choice of tissue, largely due to its extensive vascularity and its thinness, which would be a desirable feature for rapid fixation. Biochemical determinations of plasma protein concentrations had been made earlier in the 3-day chick subjected to a dose of 1400 R delivered in 15 min. Continued use of this dose was decided upon.

Tissues were fixed in phosphate-buffered (pH 7.4) 2% osmium tetroxide. After dehydration in ethyl alcohol, tissues were embedded in Epon 812, and sections were cut on a Porter-Blum ultramicrotome using glass and diamond knives.

Preliminary observations on 6-day embryonic material following a dose of 1000 R revealed a striking disruption of endothelial cells making up capillary walls. Considerable breakage of capillary walls was in evidence, together with the characteristic hemorrhage and edema associated with irradiation damage to the vascular system.

The emphasis in our ultrastructural observations has been on the 3-day chick. Chicks were given 1400 R and at various intervals following were sacrificed and the mesenteries fixed for electron microscopy. Previous data had indicated that at this dose, the chicks would show a decrease of plasma protein concentration within one hour postirradiation.⁽¹⁾ In order to ascertain that a mesentery preparation was obtained from an irradiated animal suffering from circulatory changes, it was our routine practice to take hematocrits for every animal. Furthermore, we have introduced into the vascular system, two different kinds of electron-dense tracer particles, which can be visualized in the electron micrographs. Colloidal preparations of ferritin and mercuric sulfide were introduced via the wing vein into 3-day chicks, which were then irradiated. Following appropriate intervals, tissues were fixed in osmium tetroxide.

Although our electron microscopic observations on the 3-day chick material are not yet complete, some tentative findings may be briefly summarized. Examination of animals given tracer particles in the irradiated series does not show any major alterations in the endothelial cells nor the intercellular junctions of a magnitude that will explain increased permeability of plasma protein. Many pinocytosis vesicles were observed in the endothelial cells of both unirradiated and irradiated animals. Quantitatively, there does not seem to be a difference in their numbers in both groups. Ferritin particles are commonly seen in the pinocytosis vesicles, and very rarely are the particles found free in the endothelial cytoplasm.

Majno and Palade, in a study of increased permeability in the rat cremaster muscle, have studied the effects of histamine and serotonin in increasing vascular

permeability.⁽⁶⁾ Their studies indicate that the drugs act at the intercellular junction, opening up the spaces between endothelial cells and allowing particles to pass through, thus explaining the increased permeability. In point of fact, they have published pictures of widened intercellular junctions with tracer particles in transit, but withheld by the basement membrane. Historically, it has been known for some time that histamine and serotonin are both permeability increasing agents. In our studies, we have looked at many intercellular junctions of vessels from irradiated animals, but have yet to find one that looks abnormally permeable to particles of tracer. Sometimes pinocytosis vesicles are aggregated into large bodies, called dense bodies in the literature, and we have observed a great number of particles within these dense bodies. However, at the most, we have observed 1-2 of these large dense bodies per endothelial cell, and it remains doubtful that this can be the basis of the increased permeability. Needless to say, our electron microscopic studies have yet to reveal an explanation for the physiological phenomenon of increased vascular permeability; however, they have given us some indications that our classical notions of capillary permeability are somewhat contrary to popular opinion.

Fluorescence Microscopy

Our final approach, and one in which we are still engaged in trying to work out technical difficulties, lies in the use of homologous serum proteins labeled with fluorescent dyes. We are using the fluorescent dye Lissamine Rhodamine B or RB 200, which when conjugated with serum proteins and reintroduced into the circulatory system of irradiated chicks, will serve as an excellent *in vivo* tracer. Using this experimental rationale, we have planned experiments in which we will attempt to follow the extravasation of labeled material from the vessels of the 3-day chick mesentery. We have developed a technique in which mesenteries are removed and spread and flattened on cover slips, fixed *in toto*, and subsequently prepared for fluorescence microscopy.

REFERENCES

1. Christian, E. J. B. and S. P. Stearner. Measurable indicators of permeability change in response to Co⁶⁰ γ radiation. To be published.
2. New, D. A. T. A new technique for the cultivation of the chick embryo *in vitro*. J. Embryol. Exptl. Morphol. 3, 326-331 (1955).
3. DeHaan, R. L. Organization of the cardiogenic plate in the early chick embryo. Acta Embryologiae et Morphologiae Experimentalis 6, 26-38 (1963).
4. Stearner, S. P., M. H. Sanderson, E. J. Christian and S. A. Tyler. Modes of radiation death in the chick embryo. I. Acute Syndromes. Radiation Res. 12, 286-300 (1960).
5. Majno, G. and G. E. Palade. Studies on inflammation. I. The effect of histamine and serotonin on vascular permeability: a electron microscopic study. J. Biophys. Biochem. Cytol. 11 571-605 (1961).

RESPONSE OF NEOPLASTIC MAST CELLS (P815Y) TO X-IRRADIATION.

G. Mark Kollmorgen

PURPOSE AND METHODS

Neoplastic mast cells (P815Y) grown *in vitro* are being used as a radiobiological test system to study the response of this cell line to X-irradiation. These cells grow in suspension and continue to elaborate histamine, heparin, and 5-hydroxytryptamine after extended periods of reproduction in culture.

Control cultures have a doubling time of about 12.5 hrs and are maintained within concentrations of 2×10^4 to 4×10^5 cells/cc where cell concentration does not limit growth rate.

End points being used to study damage (over the dose range 0-1000 R) include the mitotic index, the labeling indexes using specifically labeled precursors for DNA and 5-hydroxytryptamine, the total cell number, the cytological characteristics as seen with phase microscopy, and the staining characteristics using a vital stain, lissamine green.

PROGRESS REPORT

Irradiation damage, as measured by these end points, and caused by doses of irradiation up to 50 R seems to be entirely reversible. Larger doses of irradiation not only cause cell death but inflict a reversible damage that is more pronounced and more immediate. Survivors in cultures exposed to X-ray doses up to 750 R ultimately recover and again display characteristics similar to control cultures. The recovery time appears to be a linear function of X-ray dose over this dose range. Responses to higher doses of irradiation have not been clearly established.

Other studies are under way to relate the uptake of C^{14} -labeled tryptophan to the mitotic cycle and to establish the relation of uptake of this specifically labeled precursor to the elaboration of 5-hydroxytryptamine as measured by biological assays.

THE EFFECTS OF RADIATION ON CELL RENEWAL AND METABOLIC ACTIVITY

S. W. Leshner

PURPOSE AND METHODS

The reproductive and functional capacity of the rapidly-dividing, short-lived, duodenal crypt cells and the long-lived, metabolically active liver cells of the mouse and rat exposed to irradiation are being studied. This program involves single and continuous exposures, high and low doses, and X-rays, gamma rays and fission neutrons combined with the use of labeled metabolic precursors (e.g., C^{14} - and H^3 -labeled nucleotides, amino acids, etc.).

In experiments involving the duodenal crypt cells acute single exposures will be used to determine in an *in vivo* population of cells the relative sensitivity of the various stages of the generation cycle (G_1 , S, G_2 , M). In addition the rate and degree to which these cells recover from the immediate damage will be studied. The sequence of events following continuous irradiation appears to be much more involved, in that, following a single day's exposure the irradiation effects are similar to the changes produced by a single acute exposure. If the ability of the cells to divide is examined after 1 day is found that the movement of cells through G_2 is retarded, the S period is lengthened and becomes more

variable and the number of cells in mitosis is reduced. However, when the cell cycle is again studied after several days of continuous irradiation the per cent labeled mitotic figure curves are similar to control curves. However, the duration of the generation cycle is reduced, at least temporarily satisfying the increased demand for new cells. It is this compensatory behavior of the duodenal crypt cells that makes it possible for the mouse to survive even in the face of long, continued irradiation. Previous experiments have established the existence of these phenomena, while new experiments will attempt to determine the processes involved.

In experiments involving the liver we are also concerned with the immediate effects following acute doses and the so-called delayed effects following long, continued exposures. In our studies of immediate effects, we are fortunate in having the opportunity to collaborate with Dr. Van Potter and Dr. Henry Pitot. Protocols of these experiments will be available in the near future. Since liver cells have a very long life and have a tendency to become polyploid rather than divide, the accumulated damage following long, protracted irradiation can be studied.

The first in a series of experiments concerned with mitotic lag and G_2 block in the duodenal crypt cells of the mouse following single acute X-ray exposures has been completed. Results were reported at the Radiation Research meetings.⁽¹⁾ These results show that both mitotic lag and G_2 block are dose dependent. Although cells going through the G_2 phase are retarded by small doses, the block is not complete at 75 R or lower, and the number of cells entering division for the first few hours (again depending on dose) following irradiation is reduced. A comparison of these results with the effects of exposures protracted over 12 to 24 hours^(2, 3) suggests that dose rate may be an important factor. Following 150 R X-irradiation for an exposure time of 15 minutes, the proliferative cells of the intestinal crypts are completely blocked for approximately 3 hours, while similar cells in animals exposed to 220 R Co^{60} gamma rays delivered in a 12-hour interval are only temporarily slowed down, not blocked. Experiments investigating dose-rate effect are being carried out in the high-level gamma room.

Previously reported experiments⁽²⁻⁴⁾ have characterized the effects of continuous irradiations on the cell cycle of the proliferative cells of the duodenal crypts. Following a single day's exposure there is a slowing down of cells in G_2 , and fewer cells go into mitosis. However, if the irradiation continues beyond the first day, there is no apparent effect on the mitotic process, and, in fact, the generation cycle becomes shorter. The speeding-up of the generation cycle occurs at both high and low doses.^(2, 3)

Young (100 days), middle-aged (400 days), and old (825 days) mice are being studied after exposure to 12 R/day for 1, 3, and 10 days in an attempt to obtain answers to such questions as: Is there a measurable mitotic lag and/or G_2 block at low daily doses? How long after the start of exposure does the cell cycle be-

come shorter and how abruptly does it occur? On the basis of the premise that irradiation sensitivity increases with age it was reasoned that the probability of detecting small, subtle changes would be greater in older animals. Preliminary data indicate that the crypt cells are slowed down in G_2 even in the 100-day-old group, but the degree of change is greater in the old mice.⁽⁵⁾

It appears that some form of a feedback process operates, which increases the number of cells produced when the need is high, such as would be the case in animals exposed to continuous irradiation. This hypothesis is compatible with the finding of an increased cell cycle in intestinal crypt cells of germ-free mice since in the absence of bacteria, there is less attrition in the intestine; hence, the length of time the mature cells spend on the villus before being pushed off at the villus tip is increased almost by a factor of three. Our findings indicate that production of cells as a result of an increase in the cell cycle and a decrease in the proliferative population is of approximately the same magnitude.⁽⁶⁾

REFERENCES

1. Leshner, S. and A. Sallesse. Mitotic lag in duodenal crypt cells of X-irradiated mice. *Radiation Res.* 22, 208 (May 1964).
2. Fry, R. J. M., S. Leshner, A. Sallesse and E. Staffeldt. The generation cycle of duodenal crypt cells of mice exposed to 220 R Co^{60} gamma irradiation per day. *Radiation Res.* 19, 628-635 (1963).
3. 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).
4. Leshner, S., L. F. Lamerton and G. A. Sacher. Cell division in the small intestine of rats exposed to continuous gamma irradiation. *Radiation Res.* 19, 244 (1963).
5. Leshner, S. and G. A. Sacher. Generation cycle of the duodenal crypt cells of mice exposed to low-dose, continuous gamma irradiation. *Am. Zool.* 4, 271 (1964).
6. Leshner, S., H. E. Walburg and G. A. Sacher. Generation cycle in the duodenal crypt cells of germ-free and conventional mice. *Nature* 262, 884-886 (1964).

Dosimetry

NEUTRON AND MIXED-FIELD DOSIMETRY

Norman A. Frigerio, Michael H. Branson,* and Victor D. Henry

PURPOSE AND METHODS

The computation and measurement of the dose absorbed by biological specimens in neutron fields, inevitably contaminated as they are by gamma radiation, have always posed serious problems.^(1, 2) Nonetheless,

satisfactory solutions are necessary if progress in neutron radiobiology is to continue at its present pace. Furthermore, since many biological effects show sharp transitions with dose, an accuracy of $\pm 2\%$ is required for adequate dosimetry, and $\pm 1\%$ would be desirable.

The purpose of these investigations, then, is the development of convenient methods of neutron and mixed-

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field dosimetry adequate to cover the range of neutron energies from thermal to at least 15 MeV with an eventual accuracy of $\pm 1\%$. The neutron and gamma spectrometry and absolute flux measurement methods needed to supplement the above have already been developed and reported elsewhere.⁽³⁾

PROGRESS REPORT

Plane Sources

A simple expression for flux as a function of distance has long been desired. Such an expression would be useful, for example, in computing the expected dose from such sources as the converter plates at the CP-5 and Janus biological facility. Such an expression has not hitherto been available, probably because direct integration yields only open forms, and even recently new approximation and graphical methods were being employed and published.⁽⁴⁾

One can write an expression for flux ϕ as a function of distance x from a rectangular finite source of dimensions $b \times c$ as

$$\phi = \frac{S_0}{4\pi} \int_0^c \int_0^b \frac{dy dz}{x^2 + y^2 + z^2} \quad (1)$$

where S_0 is the total emission of the source. While analytical integration did not prove feasible, numerical integration with the CDC-3600 computer did.

Computation of the flux from a plate of unit area to a single point at distance x required about 3 seconds if 10^4 $dy dz$ area elements of size $10^{-2} \times 10^{-2}$, or about 150 seconds if 10^6 elements of $10^{-3} \times 10^{-3}$, were integrated.

The results obtained for squares agreed quite well with the very simple approximation obtained by assuming the flux to be equal to that from a circular disk but of equal area:⁽⁵⁾

$$\phi = \frac{S_0}{4b^2} \ln \left(1 + \frac{b^2}{\pi x^2} \right) \quad (2)$$

where b is one side of the square.

Janus Cylindrical Sources

Single panels of the Janus converter plates can be treated as above. However, the entire plates are extensive cylindrical sections of considerable curvature so that a more precise approach seemed indicated. Further, since the air path in the Janus Low Level Room⁽⁶⁾ extends to some 10 meters, correction for air interaction seemed advisable. Thus

$$\phi = \int_{z_3}^{z_m} \int_{\theta_3}^{\theta_m} \frac{R_2 \exp \{ -\Sigma [R_2^2 + R_3^2 - 2R_2R_3 \cos(\theta_2 - \theta_3) + (z_2 - z_3)^2] \}^{1/2} d\theta dz}{[R_2^2 + R_3^2 - 2R_2R_3 \cos(\theta_2 - \theta_3) + (z_2 - z_3)^2]} \quad (3)$$

where Σ is the macroscopic cross section for the media traversed and the remaining variables have the usual cylindrical coordinate significance.

Since this form did not lend itself easily to analytical integration, coordinates were transformed to Cartesian with origin in the center of the volume of interest and aligned with x the axis normal to the plate, z parallel to the plate height, and y parallel to its projected width. This was then integrated numerically by a computer program run first on an IBM 1620 then, later, transferred to an IBM 704 for greater speed. Plots of the direct flux so obtained for the 1×6 -meter Low Level

FIG. 64.—Low Level Janus direct flux, median plane ($z = 0$)

converter⁽⁶⁾ are given in Figures 64 and 65 along the central axis ($z, 0, 0$), the walls ($x, \pm 349$ cm, 0), the ceiling and floor ($x, 0, \pm 167$ cm), and the upper and lower room edges ($x, 349$ cm, ± 167 cm). The flux is expressed in units of neutrons cm^{-2} at the point $P(x, y, z)$ for a neutron output at the converter plate of 1 neutron cm^{-2} .

In addition to the direct flux originating at the converter plates reflection of neutrons from the walls will yield a very real flux of moderated neutrons.⁽⁷⁾ This was computed by a Monte Carlo method employing the IBM 704 computer and the concept of "albedo,"⁽⁸⁾ the mean fraction of incident neutrons reflected from a wall.

Results computed for the $(x, 0, 0)$ axis of the Janus Low Level Room are presented for albedo values from 0.6 to 0.9 in Figure 66 in the same manner as in Figures 64 and 65. In a separate series of experiments we have

determined that the albedo of these walls is, in fact, approximately 0.8. Thus the moderated flux at most animal positions within the room is some two orders of magnitude greater than the direct, "fission" flux as predicted previously by approximation methods.⁽⁷⁾ In consequence, the neutron energy spectra available for biological experiments do not include a fission spectrum but rather a moderated fission spectrum and, of course, a thermal spectrum which is little affected by wall reflection.

FIG. 65.—Low Level Janus direct flux, extreme planes ($z = \pm 167$ cm).

Chemical Dosimetry

Because of their ability to integrate absorbed dose with time and to assume shapes and chemical compositions similar to those of biological systems, chemical dosimeters hold considerable promise for our applications. We have, during the year, been able to reduce to convenient practice a sensitized FeSO_4 dosimeter of our own design⁽⁹⁾ and to increase the sensitivity of the Ce^{4+} dosimeter.

Two methods have been developed for the latter. The first utilizes the reaction

wherein stoichiometric quantities of radioactive mercuric ion pass into solution. An aliquot of the solution so obtained, decanted from the liquid Hg, is counted on a 4×4 -inch NaI crystal for the convenient 278-keV line of ${}^{203}\text{Hg}$. This affords a method about 100 times as sensitive as conventional Ce^{4+} spectrometry at $315 \text{ m}\mu$ and a potential precision of $\pm 0.1\%$.

The second method involves fluorimetric determination of the sodium fluorescein remaining after stoichiometric oxidation of an excess by ceric ion. This method possesses a sensitivity about 25 times greater than that of conventional Ce^{4+} spectrometry at $315 \text{ m}\mu$. It is quite simple in practice and possesses a potential precision of $\pm 1\%$. Further comparative studies of the two methods are in progress.

Finally a Monte Carlo method has been developed,

FIG. 66.—Low Level Janus reflected flux, central axis ($y = 0, z = 0$)

utilizing the CDC 3600 computer, which affords complete space-energy-dose histograms for neutrons and/or gamma rays with any of a large number of object shapes, sizes, chemical compositions, and types of radiation sources. At present this program is valid to 15 MeV for gamma rays and 0.2 MeV for neutrons, and work is under way to extend its utility to 15 MeV for neutrons.

In addition, a calorimetric method of absolute dosimetry operating at 4.2° K is described elsewhere in this report.

CONCLUSIONS

Neutron and mixed-field dosimetry appears to be feasible and potentially convenient to a precision of 1% or better, utilizing a combination of chemical and computer methods. An accuracy of the same order also appears to be feasible when chemical G values, neutron cross sections, and calorimetric calibrations become available at a corresponding level of accuracy. Current efforts are being directed toward reduction of the chemical and computer methods to convenient practice.

REFERENCES

1. Rossi, H. H. Neutrons and mixed radiations. *Radiation Dosimetry*, ed. G. J. Hine and G. L. Brownell. Academic Press, New York, 1956, pp. 667-691.
2. U.S. National Bureau of Standards. Measurement of absorbed dose of neutrons and mixtures of neutrons and gamma rays. Handbook #75. Supt. of Documents, Washington, D.C., 1961.
3. Frigerio, N. A. and D. L. Jordan. Patterns of lethality and absorbed dose distributions in mice for monoenergetic neutrons. *Biological Effects of Neutron and Proton Irradiation*. International Atomic Energy Agency, Vienna, 1963. Vol. 1, pp. 117-128.
4. Bibergall, A. A. A new graphical method of calculating the dose field of extended sources. *Reactor Sci. Technol.* **14**, 60-61 (1961).
5. Loevinger, R. Discrete radioisotope sources. *Radiation Dosimetry*, ed. G. J. Hine and G. L. Brownell. Academic Press, New York, 1956, pp. 761-762.
6. Beidelman, J. A., J. G. Condelos and W. H. McCorkle. Safety Analysis Report, Biological Irradiation Facility, Argonne National Laboratory, ANL-6643, to be issued.
7. Frigerio, N. A. and D. L. Jordan. Fast and epithermal fluxes in neutron radiobiological facilities. Argonne National Laboratory Biological and Medical Research Division Semiannual Report, January through June 1962. ANL-6723, pp. 28-32.
8. Glasstone, S. *Elements of Nuclear Reactor Theory*. Van Nostrand, New York, 1952, pp. 129-136.
9. Frigerio, N. A. Increased sensitivity for ferrous and ceric sulfate dosimetry. *Radiation Res.* **16**, 606-607 (1962).

PRODUCTION OF NEUTRONS FROM NITROGEN-17 FOR RADIOBIOLOGICAL USE

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PURPOSE AND METHODS

It has been suggested that reactor produced nitrogen-17⁽¹⁻³⁾ could serve as a useful source of effectively monoenergetic neutrons for radiobiological studies. The advantages of monoenergetic neutrons for radiobiological studies have already been discussed elsewhere.⁽⁴⁾

The delayed neutron emitter N¹⁷ decays with a 4.14-sec half-life to yield a group of monoenergetic neutrons at 1.22 MeV (60.5%) and another at 0.426 MeV (39.5%).⁽⁵⁾ Since the half-life is so short, special methods were indicated for its production and measurement.

The purpose of this investigation, then, was the study of the feasibility of using N¹⁷ produced neutrons for radiobiological experiments in conjunction with existing or proposed reactor facilities, particularly the Janus reactor.

PROGRESS REPORT

From the cross-section and yield data available^(2, 3) the coupled sequence of reactions

was chosen. Utilizing a closed microreaction and distillation system of our own design we refluxed metallic lithium (95.60 atom % Li⁶) anaerobically with H₂O (10.61 atom % O¹⁸, 0.179 atom % O¹⁷) to yield lithium hydroxide monohydrate. The latter, on titration, yielded an equivalent weight of 42.55. Theory for Li⁶O¹⁸H·H₂O¹⁸ containing 95.6 atom % Li⁶, 10.61 atom % O¹⁸ and 0.179 atom % O¹⁷ gives 42.515.

Computation, confirmed by preliminary measurements, indicated the violent energy release of some 4.8 MeV per collision of Reaction 1 would quickly afford temperatures of the order of a 1500° K or more for an irradiated sample. Accordingly a special reactor-grade graphite container (mp ≥ 4000° K) was designed and employed.

Samples of the compound in the graphite container were irradiated with thermal neutrons in the core of CP-5 for periods of 2.1 to 20.7 seconds (0.5 to 5.0 N¹⁷ half-lives) and transferred rapidly to a counting station

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via a vacuum tube "rabbit" facility. At the station counting of the N^{17} neutrons was accomplished with a battery of conventional long and Hurst proportional counters. These were calibrated between runs with a Cf^{252} fission neutron source lent by Dr. Paul Fields of the Chemistry Division.

The data obtained are presented in Table 12 converted to total N^{17} atoms present in the sample when irradiated to saturation at a flux of 4.26×10^{13} neutrons $cm^{-2} sec^{-1}$.

TABLE 12
TOTAL ATOMS OF N^{17} PRESENT IN SAMPLES OF
 $Li^6 O^{18} \cdot H_2O^{18}$ IRRADIATED TO SATURATION AT 4.26×10^{13} N $CM^{-2} SEC^{-1}$

Sample Size, g	N^{17} Content, Atoms	Specific N^{17} Content, Atoms g^{-1}
0.032.....	4.20×10^7	1.28×10^9
0.062.....	7.45×10^7	1.21×10^9
0.264.....	1.68×10^8	6.36×10^8
0.608.....	2.56×10^8	4.21×10^8
1.271.....	1.03×10^9	8.09×10^8

It is evident from the data that N^{17} concentration and, therefore, maximum neutron output, was not linear with increasing sample size. Since this appeared to be a result of self-absorption by increasing vertical thicknesses of sample, a new graphite container was designed. Instead of containing the sample in a central well 15 mm in diameter, the sample was contained in a cylindrical shell 1.0 mm thick, 40.5 mm high and with a 36-mm outer diameter. The result of runs with this container, filled by 1.271 g of compound, are included in Table 12.

From this latter experiment it appeared that containers of the order of $50 \times 50 \times 50$ mm could be designed which would hold about 2 g of sample and still afford nearly maximum neutron yield. Other experiments confirmed the theoretical prediction that under these conditions neutron yield would be proportional to thermal neutron flux.

Such a package could be irradiated for about 8 seconds (2 half-lives), transported within 2 seconds to a radiobiological facility and there employed as a neutron source for 8 seconds. With a reactor core flux of 4.26×10^{13} n $cm^{-2} sec^{-1}$ (maximum flux at CP-5) a total useful

output of about 6×10^8 neutrons would be obtained. Since a distance of the order of 10 cm from source to object would be minimal for even small biological samples, this would yield a fast neutron flux to the sample of about 5×10^6 n cm^{-2} in 8 seconds. For the neutron energies of N^{17} (6) this represents an average dose of the order of 10^{-4} rads sec^{-1} , a value much too low to be very useful. Even at a core flux of the order of 5×10^{13} n $cm^{-2} sec^{-1}$, as proposed for the Argonne Advanced Research Reactor and even if the O^{18} content of the compound were increased to 90 atom %, the maximum dose rate would still only be about 0.05 rads sec^{-1} , a value still too low to be very attractive.

Gamma contamination of the N^{17} neutrons from the reaction $O^{18}(n, \gamma)$ and from activation products in the graphite proved to be of the order of 10–20% on a dose basis. This is acceptable but also unattractive as is the production of two separate and fixed neutron energy groups by the N^{17} .

By contrast we have been able to produce truly monoenergetic beams of any desired energy from 0.03 to 1.8 MeV by use of the $Li^7(p, n)$ reaction in conjunction with a 4-MeV Van de Graaff accelerator.⁽⁴⁾ Dose rates ranged to over 1 rad sec^{-1} with gamma contamination less than 0.8%.

CONCLUSIONS

The production of monoenergetic neutron groups at 1.22 and 0.426 MeV for radiobiological use via reactor production of N^{17} has proved feasible. Nevertheless, dose rates seem limited to values too low to prove attractive, especially if compared to those available from charged-particle reactions employing Van de Graaff or other accelerators.

REFERENCES

1. Amiel, S. Private communication (1961).
2. Amiel, S. Li and Li^6 analysis by counting delayed neutrons. Israel Atomic Energy Commission Report, IA-690 (1962).
3. Amiel, S. O^{18} analysis by counting delayed neutrons. Israel Atomic Energy Commission Report, IA-691 (1962).
4. Frigerio, N. A. and D. L. Jordan. Patterns of lethality and absorbed dose distributions in mice for monoenergetic neutrons. *Biological Effects of Neutron and Proton Irradiation*. International Atomic Energy Agency, Vienna, 1963. Vol. 1, pp. 117–128.
5. Perlow, G. Delayed neutrons from N^{17} . *Phys. Rev.* 122, 899–901 (1961).
6. Rossi, H. H. Neutrons and mixed radiations. *Radiation Dosimetry*, ed. G. J. Hine and G. L. Brownell. Academic Press, New York, 1956, p. 676.

DEVELOPMENT OF MONOENERGETIC NEUTRON SOURCES FOR RADIOBIOLOGY

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PURPOSE AND METHODS

For many years investigators have been well aware of the practical and theoretical advantages to be gained by using monoenergetic neutrons in radiobiological studies. Briefly these advantages include:

1) Low contamination by interfering gamma and other irradiation.

2) The ability to establish uniformity and control of those radiation qualities of chief importance in biological effects, i.e., LET, Y and Z.⁽¹⁾

3) The ability to explore, via the prominent neutron resonances of carbon, oxygen, nitrogen, and other biologically important elements, the relative importance of these elements in radiobiology and, indeed, in the cell in general.

4) The ability to control and optimize neutron irradiations for therapeutic or exploratory purposes as in neutron capture therapy or fast neutron therapy.

Except in the less interesting neutron energy regions above 2 MeV (where neutron effects become increasingly similar to gamma effects) use of such neutrons has been deterred by technical difficulties. It is the purpose, therefore, of these investigations to develop a radiobiologically useful source of monoenergetic neutrons free of contaminating radiation and sufficiently flexible to afford rapid irradiation of typical biological objects at neutron energies from epithermal to 2 MeV.

PROGRESS REPORT

The progress made in the last few years in the development and testing of useful monoenergetic neutron sources here⁽²⁾ and elsewhere⁽³⁾ has been reported previously. Within the past year, utilizing the Li^7 (p,n) reaction in conjunction with 4-MeV Van de Graaff accelerators, we have been able to make some further progress.

Contamination-free targets of Li metal have been obtained with yields closely approaching the theoretical despite proton thickness of up to 150 keV. Spray-cooling techniques have permitted operation of rotating Cu targets at current densities in excess of 100 microamperes of resolved protons per cm^2 . Targets operated under such conditions have performed with 1-3% loss of neutron yield over 20-hour periods.

Magnetic sweeping methods have permitted spreading such beams over better than 25 cm^2 of area. Thus, when currently proposed accelerators become available (with beam currents in excess of 2000 microamperes) we hope to have targets sufficiently developed to make full use of their capabilities.

Methods of irradiating mice, mammalian cells, seeds,

human (and other) tissue phantoms, and dosimetric and spectrometric devices have been developed. In order to provide precisions adequate to the limits of our calorimetric and fission dosimetric systems ($\pm 0.1\%$) we have developed mechanical positioning systems utilizing micrometer heads, a theodolite stand and optical transits and cathetometers. These have proved capable of beam and object positioning to precisions of the order of 0.1 mm.

TABLE 13
PRELIMINARY RESULTS OF MONOENERGETIC
NEUTRON IRRADIATIONS

Test Object	CF #1 Female Mice			ANL-CF Female Mice	Seeds, <i>Avena sativa</i>
Maximum neutron energy, MeV.....	0.496	0.598	0.658	0.658	0.658
Target thickness, keV....	100	100	100	100	100
LD ₅₀ , rads....	240 $\pm$ 50	230 $\pm$ 30	210 $\pm$ 10	275 $\pm$ 50	1200 $\pm$ 150

Preliminary results obtained with some biological objects are presented in Table 13. Others have already been presented elsewhere.⁽²⁾

Finally, our examination of various nuclides for response to fast neutrons has shown that the activation of Rh^{103} to 57-minute Rh^{103m} occurs only above neutron energies of 0.04 MeV. Preliminary cross-section and counting data obtained indicate that foils of Rh can serve as very useful and precise monitors of fast neutron flux during radiobiological irradiations.

CONCLUSIONS

Present facilities are capable of delivering precise, well characterized doses to biological objects at dose rates in excess of 1 rad sec^{-1} , at neutron energies from 0.03 to 2.7 MeV and spectral widths of some 100 keV. With advancing accelerator development it should soon be possible greatly to increase these dose rates and decrease the available neutron energy and spectral widths to a few keV.

REFERENCES

1. Rossi, H. H. Correlation of radiation quality and biological effects. *Ann. N.Y. Acad. Sci.* 114, 4-31 (1964).
2. Frigerio, N. A. and D. L. Jordan. Patterns of lethality and absorbed dose distributions in mice for monoenergetic neutrons. *Biological Effects of Neutron and Proton Irradiation*. International Atomic Energy Agency, Vienna, 1963. Vol. 1, pp. 117-128.
3. Bateman, J. L. and V. P. Bond. The effects of radiations of different LET on early responses in the mammal. *Ann. N.Y. Acad. Sci.* 114, 32-47 (1964).

ABSOLUTE NEUTRON DOSIMETRY BY CALORIMETRIC MEANS

Paul Mitacek, Jr., and Norman A. Frigerio

PURPOSE AND METHODS

The purpose of this project is to provide a method to measure the absolute neutron dose at radiation dose rates provided by the Janus reactor. These measurements will be part of the over-all dosimetry on the Janus reactor necessary to characterize its radiation for the biological radiation experiments of this Division.

A calorimetric method using a calorimeter operating at liquid helium temperature (4.2°K) was found to be the most suitable method. The design and development of a radiation measuring calorimeter operating at 4.2°K was a major portion of this project to date and will be described in the following paragraphs.

PROGRESS REPORT

The definition of a rad (100 ergs of energy absorbed per gram of irradiated matter⁽¹⁾) makes possible absolute dosimetry by calorimetric measurement of the energy deposited in an irradiated sample. For the study of biological systems, measurements of 100 to 1,000 rads to an accuracy of 1% or better are necessary. Two calorimetric methods which have the desired sensitivity to measure the energy inputs corresponding to 100 to 1,000 rads were investigated and evaluated as to their applicability to this project.

One of these methods is the Calvet microcalorimeter.⁽²⁾ The essential feature of this method is a calorimeter with two sets of multiple thermocouple junctions uniformly fixed about its surface with the other ends on a reference surface. One set of thermocouples is used to monitor the temperature change of the calorimeter. When the calorimeter is irradiated, the energy put into the calorimeter is balanced by Peltier cooling in the calorimeter caused by a measured current in the second set of thermocouples. The Peltier cooling, measured accurately by electrical means, balances the radiation heating when the temperature of the calorimeter remains constant and thus is a measurement of the radiation dose.

Radiation sources inside the calorimeter can be measured by this method without any modification of the present apparatus design. The method could not be used for external radiation sources because of the perturbing effect on the radiation of the thermocouples surrounding the calorimeter. No modification of the method was found to be feasible for measurements of external radiation sources. Thus, the method is not applicable for this project.

The second calorimetric method investigated was the

carbon calorimeter designed and developed by Laughlin.⁽³⁾ A carbon disk is used as a calorimeter and absorbing material. It is surrounded by a thermal shield whose temperature is monitored and controlled by electrical heating. For a dose of 100 rads, the temperature rise at room temperature is about 10^{-4}°C . Thus, very sensitive temperature measurement is necessary in order to measure a 10^{-6}°C temperature change necessary to give 1% accuracy of results. This sensitivity is achieved by incorporating a thermometer with -4% change of resistance per degree in one arm of a Wheatstone bridge system. As the temperature in the calorimeter rises, the resulting bridge offset produces a signal which is amplified by electronic means. The heating of the calorimeter by radiation is related to energy and rads by comparing the electrical heating necessary to produce the same temperature rise.

This method was extensively investigated as to its applicability to this project by building a model of the calorimeter and studying its characteristics as well as by visiting the Sloan-Kettering Laboratory and consulting with Dr. Laughlin on the latest improvements. The carbon calorimeter has been developed to its fullest capacity, and no major improvements in its accuracy and operating characteristics are possible. Because of the necessity of the order of 10^{-6}°C and the thermal isolation necessary to produce this thermal equilibrium, the apparatus lacks flexibility and operating characteristics desirable and necessary for this project.

The liquid helium temperature calorimeter was designed to overcome these limitations. This technique has not been previously employed in radiation calorimetry. The advantages of operating a calorimeter at 4.2°K rather than at room temperature are derived from the nature of matter at low temperatures. Four distinct advantages occur:

- 1) Heat capacities decrease with temperature and for most materials are about one thousandth as large as at room temperature. Thus, a radiation dose of 100 rads, which produces a temperature rise at room temperature of about 10^{-4}°C , produces a 0.1° rise at liquid helium temperature. With this magnitude of temperature rise, the attainment of thermal equilibrium to 0.1% and the elimination of thermal gradients in the calorimeter are no longer problems.

- 2) Thermal isolation is simpler, since the energy transfer between calorimeter and thermal surroundings by thermal radiation is smaller by a factor of 10^{-6} for the same temperature gradient. The constant tempera-

ture environment around the calorimeter can be automatically provided without monitoring or control by evaporating liquid helium. Also, the presence of large amounts of thermal shielding material which perturb the radiation field is not necessary.

3) The liquid helium cryopumps the insulating vacuum spaces between the calorimeter and its surroundings so that continual mechanical vacuum pumping is not necessary during measurements. This feature increases the portability of the apparatus and allows the apparatus to be used in large radiation dose fields such as the Janus reactor dose rooms without the presence of a radiation field perturbing vacuum system.

4) The larger temperature rise coupled with the use of germanium crystals with a resistance change of 50% per degree at 4.2° K makes it possible to measure the temperature rises directly with a potentiometer system.

This amplifies and increases the reliability of the temperature measuring system.

Figure 67 is a drawing of the low-temperature calorimeter system Mark III.⁽⁴⁾ Operation consists of pumping out the vacuum spaces through the pumping valves and then placing liquid nitrogen and liquid helium in their containers. The calorimeter is thermally shunted and cooled down to liquid helium temperature by the thermal switch. It is then thermally isolated by raising the thermal switch with the thermal switch valve. The resistance of a temperature sensitive element, a single crystal Minneapolis Honeywell germanium resistance thermometer, Series II Model, is measured with a potentiometer. After the calorimeter is irradiated, the resistance is again measured. The resistance change is calibrated in energy units by measuring the amount of electrical resistance heating necessary to produce the

FIG. 67.—Liquid helium calorimeter and cryostat assembly

same resistance thermometer change. From this the absorbed dose in rads is calculated.

The proper size, shape and composition of the radiation absorbing material used in the calorimeter were also investigated in this project. Two different physical types of calorimeter were designed and built to obtain two different types of dose measurements. The first type was of the size and approximate shape of a mouse. From this type of a calorimeter the average absorbed dose is obtained for a mouse phantom. To obtain the locally absorbed dose without any attenuation of the radiation by the absorber a calorimeter of an area 4 cm by 4 cm and a thickness of 0.3 cm perpendicular to the radiation field was built. By calculation it was shown that this thickness of tissue equivalent material attenuated the neutron radiation of one MeV energy by 1% and was thick enough to make the effect of secondary particle buildup negligible.

The composition of the calorimeter is significant for two reasons. One reason is the absorbed dose in rads depends upon the absorbing material. The absorbed dose is of interest in tissue, and materials of neutron tissue equivalent properties are the proper materials for the calorimeter. The second reason is that all of the energy deposited in the absorbing material does not appear as heat, but some of it is utilized in endothermic chemical bond breaking to form free radicals. This effect must be known and corrected. Compounds with benzene rings in them are found to be more stable to radiation damage and free radical formation than aliphatic systems.⁽⁵⁾ The effect of irradiating these materials at 4.2° K is not known and will have to be studied further as part of this

project. Biphenyl is the compound presently being used as the best absorbing material for neutron absorption. At room temperature, 0.4% of the energy deposited in it is used up in free radical formation.⁽⁶⁾

Preliminary measurements have been made with the calorimeter using the Co⁶⁰ source. At a dose rate of 100 rads per minute and a total dose of 400 rads the calorimeter was sensitive to a dose of 0.5 to 1 rad for a precision of 0.2%.

CONCLUSIONS

A calorimetric method has been designed and developed to measure the absolute neutron dose at the neutron radiation facilities of the Janus reactor. This method will be operational in time to participate in the overall dosimetry on the Janus reactor.

REFERENCES

1. International Commission of Radiological Units, 1953. Brit. J. Radiol. 27, 243 (1954).
2. Calvet, E. *Experimental Thermochemistry*. Interscience, Wiley, New York, 1962. Vol. 2, ch. 17.
3. Laughlin, J. S. and S. Genna. Calorimetric methods. *Radiation Dosimetry*, ed. G. H. Hine and G. L. Brownell. Academic Press, New York, 1956, pp. 411-453.
4. Mitacek, P. The measurement of absorbed doses of radiation by means of a calorimeter operating at liquid helium temperatures. Proc. 19th Annual Calorimetry Conference, Washington, D.C., 1964, in press.
5. Kohnlein, W. and A. Muller. Absolute yield measurements of radiation-produced radicals by electron spin resonance. Phys. Med. Biol. 6, 599 (1961).
6. Halg, W. and T. Schaub. Diphenyl cooled, heavy-water moderated, natural uranium reactor prototype. Proc. Second United Nations Intern. Conf. on Peaceful Uses of Atomic Energy. United Nations, Geneva, 1958. Vol. 9, pp. 88-98.

DETERMINATION OF ABSORBED DOSE IN PHANTOMS OF VARIOUS SIZES IRRADIATED WITH X-RAYS

Frank S. Williamson

PURPOSE AND METHODS

This study was planned as a logical extension of that undertaken by Sinclair using cobalt-60 gamma radiation.⁽¹⁾

The method employed follows that of Sinclair in that cylindrical polyethylene bottles filled with ferrous sulfate dosimeter are used as phantoms, and absorbed dose is determined by measurement of the ultraviolet absorption by ferric ion with a Beckman spectrophotometer.

For X-rays of 250 kvp it is necessary to use a dosimeter solution of 0.1 N in H₂SO₄ so that the mass energy absorption coefficient is similar to that of muscle.⁽²⁾

PROGRESS REPORT

A practical problem in applying this technique to X-ray measurements is the relatively low dose rate available from X-ray machines. Typically, at 1 meter from a 250-kvp Maxitron, using filtration of 0.25 mm Cu + 1.0 mm Al, and 30mA current, dose rates of 38 R/min are obtained. Hence, for a useful exposure of 6000 R, periods of two and one-half hours are needed. For harder filtration and greater distances from the target, much longer exposures are required. Only one phantom can be exposed at any one time, so the irradiation time require-

ments become quite impossible for X-ray machines which are already fully utilized.

As a consequence, the possibility was explored of reading the uv absorption at a wavelength of 224 m μ , rather than 304 m μ .⁽³⁾ This almost doubles the over-all sensitivity of the dosimeter. In extensive tests it was found that the method was practicable when the dosimeter was contained in ideal, inert vessels, but that apparently similar containers of polyethylene could introduce some unknown factor. Ultraviolet absorption at 224 m μ wavelength showed marked sensitivity to this factor. To borrow an analogy from electronics, the "signal-to-noise ratio" was inferior to that at a wavelength of 304 m μ . In this type of experiment the containers have to be selected for their dimensions, and any deficiencies of performance must be tolerated.

The current practice is to use the uv absorption at 304 m μ wavelength and to irradiate the phantoms with 10,000 R exposure overnight. The dosimeter uv absorption is read the next morning.

Some variability in the first series of results prompted an investigation of the change in the uv absorption of the solution after irradiation. This was found to vary with the particular container used, and the uv absorption could increase by as much as a few per cent in 24 hours.

Work has started on actual phantom measurements with corrections applied for change in uv absorption

with time after irradiation. Exposures are made with the phantom flanked by two monitoring ferrous sulfate dosimeters at the same distance from the X-ray target and 20 cm from the center of the phantom. The scatter contribution from the phantom at the monitoring points has been determined with an ionization chamber. The absorbed dose at the proximal and distal surfaces of the phantom is inferred from further ionization chamber measurements.

The ionization chamber used is air filled and has an air equivalent plastic wall of 1 mm thickness and 5/16 inches external diameter. It is provided with a guard ring and a low-noise coaxial connector to a vibrating reed electrometer. The chamber was specially designed and constructed by Dr. Francis R. Shonka of St. Procopius College, Lisle, Illinois, whose contribution to this project is a pleasure to acknowledge.

REFERENCES

1. Sinclair, W. K. Absorbed dose in biological specimens irradiated externally with cobalt-60 gamma radiation. *Radiation Res.* **20**, 288-297 (1963).
2. Shalek, R. J., W. K. Sinclair and J. C. Calkins. The relative biological effectiveness of 22-Mevp X-rays, cobalt-60 gamma rays, and 200-kvcp X-rays. II. The use of the ferrous sulfate dosimeter for X-ray and gamma ray beams. *Radiation Res.* **16**, 344-351 (1962).
3. Scharf, Karl and Richard M. Lee. Investigation of the spectrophotometric method of measuring the ferric ion field in the ferrous sulfate dosimeter. *Radiation Res.* **16**, 115-124 (1962).

GAMMA DOSIMETRY

Frank S. Williamson

PURPOSE AND METHODS

Measurements have been made of the relative intensities of the nominal 100-, 6000-, and 12,000-curie cobalt-60 sources in the High Level Gamma Room in connection with the dose-rate experiments of E. L. Powers. Two ionization chambers were used, one of 50-cc volume, air filled, with an approximately air-equivalent lining, the other of 0.3-cc volume, also air filled, with an air equivalent wall and fitted with a buildup cap. Currents from the chambers were measured using electrometer amplifiers and high value resistors. For a pair of readings from which a ratio was calculated, the same high value resistor was used. Thus the values of these resistors did not need to be known, and only the electrometer range accuracy was relied upon. All measurements were at points in the mid-source horizontal plane.

PROGRESS REPORT

For 12,000/6,000 at distances of 10, 55, 72, and 100 inches the mean ratio was 4.123, with a maximum deviation

of 1.1%. For 6,000/100 at distances of 55 and 72 inches the mean ratio was 47.24, with a maximum deviation of 0.6%. At 10 inches the ratio 6,000/100 was 49.46 and at about 1 inch was 63.94. This seems to indicate the effect of known inhomogeneity in the "100" source. Measurements of such small distances from the source tube lack reproducibility, however, and the investigation will be continued using more accurate locating arrangements for the chamber.

The 0.3-cc chamber, constructed for the X-ray Absorbed Dose project by Dr. F. R. Shonka, proved to have excellent saturation characteristics at close distances and dose rates of 30,000 R/min. Thus a detailed survey close to the "12,000" source can be made.

Work has been started on the use of lithium fluoride thermoluminescent dosimeters for *in vivo* dosimetry. Subcutaneous implants have been made in dogs to determine the surface dose during an 11-day irradiation using cobalt-60 gamma rays.

LET SPECTROMETRY

Frank S. Williamson

PURPOSE AND METHODS

In the quarter century or so in which radiobiological investigations have been conducted using fast neutrons as a source of radiation, the determination of the radiation dose has presented both conceptual and practical difficulties. At the present time it is considered good practice to measure the absorbed dose for the tissue composition of interest and also the energy spectrum of the neutrons used. Failing a measurement of absorbed dose, the neutron fluence (time integrated flux) may be determined and absorbed dose calculated. These data form a solid foundation on which any future reappraisal of the experiment can be based.

Where possible, and as technological advancement permits in individual cases, the distribution of absorbed dose within the irradiated material must be determined. These steps are essentially practical, inevitable, and undoubtedly of great value. However, the determination of Kerma or even absorbed dose absolutely to better than 1% is by no means the ultimate in sophistication.

Most experimenters would agree that the spectrum of LET (linear energy transfer) for the particular radiation used should be specified, and this is sometimes calculated from the neutron energy spectrum, though it would be of great advantage to measure LET spectra directly. Harald H. Rossi has for a number of years had a device with this capability. This consists of a special spherical counter with proportional characteristics, used with a tissue equivalent gas filling. The output pulses are analyzed using a pulse height analyzer, and analytical operations performed on the pulse height spectrum to determine the LET spectrum.

More recently Rossi has proposed the stimulating concept which he calls "Z" dose. He has described the whole subject in detail in many published works, but a very brief explanation would not be out of place here. When a mass of tissue is irradiated by fast neutrons it is

the atomic nuclei recoiling from elastic collisions with the neutrons which produce densely ionizing tracks through the tissue (high LET). By contrast, the same absorbed dose of X- or gamma radiation produces a much greater number of electron tracks having longer ranges and a low density of ionization (low LET). If we accept that biological damage is caused by the deposition of energy from a track in microscopically small volumes (targets) less than 1 micron in linear dimension, then it becomes apparent that the difference between these two cases is as follows: the X- or gamma radiation gives rise to a large number of targets, each receiving a modest energy deposition, while the neutron radiation gives rise to a much smaller number of targets, each receiving a larger (orders of magnitude) energy deposition.

The energy deposited in a single spherical target of chosen diameter in one "event" is called by Rossi local energy density "Z," having the dimensions of absorbed dose and expressed in units of ergs/g. The probability of the event is expressed by $P(Z)$.

It is convenient actually to measure these parameters directly in a very elegant manner using the same spherical counter apparatus previously mentioned with a different analysis of the pulse-height spectrum.

PROGRESS REPORT

Funds for purchase of the equipment became available in June, 1964, and the 2-in. spherical counter, 400-channel Packard pulse height analyzer and ancillaries have been delivered. Other items are in course of design and construction. We propose to use the method rather widely to determine LET spectra on all radiation sources, both neutron and gamma-ray, and to attempt to correlate the "Z" parameter with biological effect in a wide variety of situations.

Professor Rossi and his collaborators continue to give us unstinting help and encouragement, which we gratefully acknowledge.

Neutron and Gamma-Ray Toxicity Studies (Janus Program)

THE JANUS PROGRAM FOR INVESTIGATING THE TOXIC ACTIONS OF NEUTRONS AND GAMMA RAYS

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PURPOSE AND METHODS

This program will focus the efforts of an interdisciplinary research team on the description and analysis of the toxic actions of neutrons and gamma rays on mammals. The program will be built around two major radiation facilities in the Biology Division—the biological research reactor Janus and the gamma irradiation rooms. Since both facilities produce broad radiation beams, most, if not all, irradiations will be whole-body exposures.

The major test object will be the laboratory mouse, but considerable use will be made of other small vertebrates, including fish, birds, rodents, and primates. The efforts to introduce a new fish species, *Cynolebias adloffi*, are described separately. Two rodent species that will certainly be used are *Peromyscus leucopus* and *Mastomys natalensis*. Preliminary work with these species and others is described separately. Some research projects within the program are described briefly below. These projects will be cross-linked in various ways, so the separate headings are in some degree arbitrary.

Conjoint Study of the Acute Radiation Syndrome

In this study the acute dosage- and time-lethality curves for a hybrid mouse will be determined with greater accuracy, and subsamples will be drawn from the same groups for determination of the cell populations in the radiosensitive tissues. The intent is to establish quantitative relationships between tissue cell depletion and specific disease risks, such as hemorrhage, bacteremia, anemia, etc. In particular, the relation of the killing curve for cells and the killing curve for the entire animal will be sought. This will be the foundation of much of the work described below. The role of intestinal flora in the acute radiation syndrome is being examined. Germfree animals and animals with endocrine ablations will be used to facilitate the study of responses of the cellular renewal system.

Repair and Recovery at the Level of the Cell in Vivo and the Whole Animal

This is an extension of the techniques described above to paired and multiple exposures. Extensive use will be

made here of the phenomenon of colony formation in the spleen. This problem of recovery is a fascinating area of investigation and one of the major problems of the Janus program. Additivity studies are closely related to recovery studies and will be carried out concurrently.

Daily Dose Lethality and Age Dependence

The detailed relation of survival time to daily dose of gamma rays that was established from the LAF₁ mouse in the gamma-ray toxicity program was informative in a number of ways. Several modes of injury were identified and characterized quantitatively. Radiosensitivity profiles were determined for several mouse strains and hybrids. A similar, but more economical program will be carried out to compare neutrons with gamma rays. The age dependence of sensitivity to neutrons and gamma rays will be determined by single-dose and duration-of-life exposures. The data will be used to test hypotheses about the mechanism of radiation injury in mammals.

Life Shortening and Late Pathological Effects

These effects will be examined with single, multiple, and duration-of-life exposures. Interest will center on the shapes of the dosage-survival time curves for fast neutrons and gamma rays, on the relation of RBE to dose and dose rate, and on the dependence of life shortening effectiveness on time factors of the exposures (i.e., total dose, number of fractions, interval between fractions).

Effect of Physical Environment during Irradiation

This will be primarily an investigation of the effect of the anoxic condition in mice on the sensitivity to acute and late effects. This is an extremely important area of investigation, but it poses serious technical problems because of the long neutron exposure time necessitated by the low dose rate attainable. Other physical factors (temperature, oxygen tension, nitrogen or rare gas pressure, etc.) can be studied in *Cynolebias*.

Cell Renewal Processes in Intestinal Epithelium and Cornea

These two tissues are especially favorable for the investigation of different aspects of cell division and dif-

ferentiation. Techniques now available or under development will be used to study the action of fast neutrons and gamma rays.

Effect of Ionizing Radiations on Growth of the Neoplastic Mast Cell in Liquid Suspension and in the Peritoneal Cavity, and on the Production of a Specific Secretion, 5-Hydroxytryptamine

The neoplastic mouse mast cell grows well in liquid suspension and as an ascites tumor. Its response to X-irradiation has been followed in detail, and it should be a suitable test object for neutron-gamma comparisons. It is also virtually unique in that it retains the ability to synthesize serotonin. The use of bioassay procedures and labeled precursors, together with techniques for producing partial synchrony of cell division, should provide a good, simple model system for studying the effects of ionizing radiations on the growth and biochemical activity of a mammalian cell.

Relation of Cytogenetic Effects of Ionizing Radiations to Their Carcinogenic Action

This is a program that seeks to elucidate the mechanism of radiation carcinogenesis by the combined application of two new research tools. It proposes to karyotype a large number of irradiated and unirradiated cells—perhaps a million nuclei in all. The radiation doses would be in the range from 100 R down to 10 or even 1 R if this proves feasible, and the results would permit construction of a dose-effect curve for chromosome aberrations. The cells would come from a small rodent species so that 1000 to 10,000 or so could be followed at similar and higher doses to determine incidence of various neoplasms. The dose-response curves for neoplasms could then be compared with that for chromosome aberrations. In addition, the karyotypes of the neoplastic cells would be determined.

The best species for this project is the multimammate mouse, *Mastomys (Rattus) natalensis*, which has a better karyotype than the usual laboratory species. (See report on the comparative physiology and anatomy of radiosensitivity and aging.)

The karyotyping part of the project is feasible only because of the cooperation of the Applied Mathematics Division, which has developed a computer program for producing chromosome classifications. The system already works well, and the impetus given by a large re-

search project of this kind is just what is needed to bring it to full development and demonstrate its capabilities.

Long-Term Environmental Factors

Little is known about the relation of chronic radiosensitivity to environmental factors such as temperature, humidity, population density, etc. At low levels of radiation exposure paradoxical effects are sometimes observed. These effects are too large and too frequent to be sampling deviations, so it must be concluded that they result from interactions of unknown nature between the radiation effects and other environmental effects. Since the Janus program gives much emphasis to the long-term effects of small doses, it is essential that these phenomena be understood. The program has, therefore, specified the construction of two rooms with temperature control permitting operation down to 10° C. These rooms and two additional rooms will have auxiliary lighting systems and controls installed so that they can be operated on reversed or otherwise altered light-dark cycles.

Behavioral Studies

A laboratory for behavioral research is now being equipped. There will be two main areas of interest: 1) investigation of the ability of animals to detect low levels of ionizing radiations, as measured by avoidance or escape behavior; 2) a program of behavioral genetics that will look for relationships of psychological traits to radioresistance and longevity.

The above projects give a rough schema of the Janus research program. It cannot be set up all at one time, of course. The first stages will be concentrated on the acute radiation response, especially the first two projects described.

The program will also be oriented toward bringing in participating investigators who can apply their own experimental procedures to groups of animals that constitute a balanced sample from the over-all experiment design. In this way many outside investigators will gain access to a unique resource, and the amount of information produced will be greater and more varied than could be produced from a completely in-house program.

PROGRESS REPORT

Only a few phases of the program are operating at present, so there is little material progress to report.

NEUTRON DOSIMETRY: THE JANUS REACTOR

Frank S. Williamson and Martin C. Schumacher*

PURPOSE AND METHODS

The reactor diverged for the first time on August 12, 1964. Starting on August 21, with the full cooperation of the Reactor Operations team, preliminary data were obtained on the neutron radiation in the two exposure rooms.

FIG. 68.—Neutron flux distribution across the high-dose face of Janus. X—X, slow neutrons without converter plate, all lead in place; ●—●, as above, but central 24-inch section of lead removed; ○—○, with converter plate and all lead in place, fast neutrons; Δ, same, single slow neutron point.

The reactor was still under test at very low power (estimated 1 watt or less); consequently observations were limited to measurements of neutron flux using a long counter, mostly in the High Level Exposure Room.

* Industrial Hygiene and Safety Division.

PROGRESS REPORT

For the present purpose fast neutrons will be taken to mean those to which a moderated long counter is sensitive, and slow neutrons those capable of detection with a bare BF₃ counter tube.

Measurements were made at the center of and immediately adjacent to the lead brick shield wall at each of the two exposure faces. The ratio between the fast neutron fluxes, under these conditions, was determined:

$$\frac{\text{High level flux}}{\text{Low level flux}} = 1003.$$

We consider that this determination was accurate to $\pm 10\%$ or better. We do not expect the ratio to change as the power level is raised. This number can, therefore, be taken as the ratio between the maximum fast neutron fluxes at any reactor power level.

Measurements were made of fast and slow neutron flux at three points down the center of the High Level Exposure Room, 1 meter above the floor. The first position, with the front of the moderated long counter touching the lead brick shield wall, is 75 cm from the converter plate. The rear wall of the room is 262 cm from the lead wall and 337 cm from the converter plate.

Distance from Converter Plate, Cm	Fast Neutron Flux, n cm ⁻² sec ⁻¹	Slow Neutron Flux, n cm ⁻² sec ⁻¹
75.....	394	14.9
190.....	120	15.8
280.....	84	16.3

It will be apparent that, although the fast neutron flux falls to about one-fifth in the length of the room, the slow neutron flux remains fairly constant.

A traversal of the counter across the lead brick face in the High Level Exposure Room was performed. The height chosen was that of the center of the face. The results are shown in Figure 68 for four conditions.

THE EFFECT OF PURINE COMPOUNDS ON MUTATION IN BACTERIA

Harold E. Bendigkeit

PURPOSE AND METHODS

Caffeine and other methyl xanthines are effective mutagens for bacteria.^(1, 2) However, very little is known about the mechanism by which mutations occur in the presence of these purines. For this reason, we are studying both the manner in which caffeine is accumulated in cells and the effect of antimutagenic purine compounds on caffeine accumulation. An investigation of the kinetics of concentration and the location of bound caffeine in cells should aid in elucidating the mechanisms of caffeine-induced mutation and antimutagenic activity of related purine compounds.

The kinetics of accumulation of caffeine within cells of *Escherichia coli* strain B/r/1, try (tryptophan requiring) are being studied using C^{14} -caffeine as a tracer. Radioactivity of cells is measured either with a low-background Geiger counter or a liquid scintillation counter.

The concentration of free caffeine within cells is obtained by measuring the amount of activity which is removed from cells by washing. Bound caffeine levels in cells are measured from the remaining radioactivity in cells.

Variations in the behavior of discontinuous (flask) and continuous (chemostat) cultures are being measured at several temperatures. All cultures are grown in defined glucose-salts media with added tryptophan. Limitation of a nutritive, where used, has been of glucose or tryptophan.

Mutations from sensitivity to resistance to bacteriophage T5 are measured by a standard procedure.⁽²⁾

Extracts of cell components are made following the procedure of Roberts et al.⁽³⁾ Separation of components within the nucleic acid fraction has been accomplished using high-voltage paper electrophoresis in formate-citrate buffer at pH 2.

PROGRESS REPORT

Total concentration of caffeine in cells reaches that of the surrounding medium within ten minutes after exposure. All caffeine can be washed out of cells exposed

in the absence of an energy source. No mutations are observed under these conditions.⁽⁴⁾

In the presence of an energy source, however, cells accumulate caffeine which is not removed by washing. The initial rate of accumulation and amount bound are proportional to the caffeine concentration in the medium. The maximum concentration of bound caffeine reaches about 10 per cent of that in the medium,

FIG. 69.—Levels of bound caffeine in two glucose-limited chemostats. τ is the time interval for an e-fold increase in cell number.

some 5×10^5 molecules per cell in $1 \times 10^{-3} M$ caffeine solution. The initial rate of binding in chemostat cultures is independent of growth rate or limiting nutritive, and maximum levels of bound caffeine are reached within three hours (Figures 69 and 70).

Purine ribosides showing antimutagenic activity (adenosine, guanosine, and inosine) decrease both initial rates of accumulation and final levels of bound caffeine. These reductions are proportional to the concentration of antimutagen and are parallel to the corresponding reductions in mutation rates. For example, adenosine at 1 mg per liter causes a four-fold reduction

in mutation rate and initial accumulation rate of bound caffeine. Guanosine gives two-fold reductions at the same concentration. The concentration of unbound caffeine in cells is not reduced by the antimutagens. Thus bound caffeine appears to be the relevant component in caffeine-induced mutagenesis. Purine and pyrimidine compounds not affecting the mutagenic activity of caffeine have no appreciable effect on accumulation or final level of caffeine bound in cells.

The kinetics of accumulation of bound caffeine indicate a noncompetitive inhibition of caffeine binding sites by the antimutagens. Caffeine is not displaced to any appreciable extent by the addition of enough antimutagen to prevent binding of additional caffeine. In continuous culture under such conditions, the rate of decrease of bound caffeine in cells agrees with the rate of dilution expected by growth and division of cells (Figure 70).

Binding of caffeine appears to be through energy-requiring reactions. The initial rate of binding in flask cultures is growth-rate and temperature dependent with a Q_{10} near 2. Metabolic inhibitors such as azide blocks binding, while chloramphenicol, an inhibitor of protein synthesis, does not.

The majority (two-thirds) of bound caffeine in cell extracts is found with the nucleic acid fraction. However, this caffeine is in unbound form among the partially hydrolyzed nucleic acid components. Similarly, when cells are treated with mitomycin C, an agent acting to disrupt DNA, very little bound caffeine accumulates.

Since extraction procedures separate nucleic acids from residual protein components by liberating acid-

soluble nucleic acid constituents, the possibility exists that bound caffeine is at least partially associated with protein. An attempt to locate bound caffeine among the major constituents of cells by electrophoretic separation of gently disrupted cell contents is in progress.

FIG. 70.—Levels of bound caffeine in two tryptophan-limited chemostats. τ is the time interval for an e-fold increase in cell number.

REFERENCES

1. Novick, A. Mutagens and antimutagens. *Mutation*. Brookhaven Symp. in Biol. 8, 201-214 (1955).
2. Kubitschek, H. E. and H. E. Bendigkeit. Latent mutants in chemostats. *Genetics* 46, 105-122 (1961).
3. Roberts, R. B. et al. Studies in biosynthesis in *Escherichia coli*. Publ. 607, Carnegie Institute of Washington. Washington, D.C., 1955, pp. 13-16.
4. Glass, E. A. and A. Novick. Induction of mutation in chloramphenicol-inhibited bacteria. *J. Bacteriol.* 77, 10-16 (1959).

THE CONTROL OF MACROMOLECULAR SYNTHESIS IN BACTERIA

Richard E. Ecker

PURPOSE AND METHODS

These investigations were initiated to study the basic interactions of the constituent macromolecules of a living cell which result in growth and/or growth control. The ultimate goal of this work is the synthesis of a model of cellular growth control in which the role of each class of participating macromolecule is understood in relation to all other classes of such molecules and in relation to its own physical and chemical properties.

For ease of manipulation and relative simplicity of function, bacterial systems have been employed. Two general types of experimental procedures have been followed: a) Balanced growth studies⁽¹⁾ in which the cell composition is studied as a function of the rate of synthesis; and b) unbalanced growth studies⁽¹⁾ in which the

kinetics of specific processes can be investigated independently. Kinetic and chemical analyses have been carried out by standard procedures of radioisotope incorporation and/or analytical biochemistry.

PROGRESS REPORT

Because this investigation was initiated at this site only recently, progress has been limited to reestablishment of the physical arrangements necessary for this work and repetition of a number of earlier experiments.

REFERENCE

1. Ecker, R. E. and M. Schaechter. Bacterial growth under conditions of limited nutrition. *Ann. N.Y. Acad. Sci.* 102, 549-563 (1963).

THE INDUCTION OF CHROMOSOME ABERRATIONS IN PIG KIDNEY CELLS BY FAR-RED LIGHT

Solon A. Gordon, Agnes N. Stroud, Nancy A. Munro, and Chen H. Chen

PURPOSE AND METHODS

We have been investigating the potentiating effect of far-red light on the chromosome damage induced by X-rays. In one series of experiments an enhancement of aberration frequency in ascites tumor cells (Krebs-2) was found to be correlated with the impairment of phosphorylative capacity.⁽¹⁾ For several reasons ascites cells are unsatisfactory material, and we turned to pig kidney cells. Monolayer cultures of the kidney cells exposed only to far-red light possess very high aberration frequencies compared to those unexposed. The present report describes this phenomenon.

PROGRESS REPORT

Experiment I

Leighton tubes containing diploid pig kidney cells in monolayer were X-irradiated (50 R) 18 hours after subculture. Preceding and following the X-irradiation the cells were exposed to light, between 730 and 750 m μ in wavelength, from a high energy monochromator of the Withrow type. The total incident energy in the far-red was 3.9 joules cm⁻². Three controls were used: one exposed the cells only to X-radiation, another only to far-red light, and a third to no radiation. Metaphase aberrancies were scored at various times after irradiation. The results are shown in Figure 71.

No enhancement was noted. Furthermore, exposure only to the far-red gave rise to an aberration frequency

FIG. 71.—The effect of far-red and X-radiation, singly and combined, on the percentage breaks and exchanges occurring in pig kidney cells at various times after irradiation.

equal to or greater than that induced by the X-radiation. Following both types of radiation, average percentage frequencies of about 100 chromatid breaks and 50 exchanges were observed. The maximum aberration

frequency induced by far-red light alone appeared to occur about a day later than the maximum observed for cells exposed to X-rays alone or in combination.

Experiment II

The dosage-response relation for the pig kidney cells was then determined. Eighteen hours after subculture Leighton tubes were exposed to various irradiances of far-red light in the monochromator. The cells were first equilibrated 15–30 minutes to either 17°, 25°, or 37° C. They were irradiated at these temperatures and then returned to the dark at 37°. Thirty-six hours later the cells were treated with Colcemide and 2 hours later fixed for the scoring of metaphase aberrations. Response curves for 17° and 25° are shown in Figure 72.

It may be seen that the maximum aberration frequency occurs at irradiances of ca. 50 millijoules cm⁻².

FIG. 72.—The effect of various exposures to far-red light at 17° and 25° on metaphase aberrations.

Approximately the same order of irradiance was maximum at 17° and 25°. Though not shown in the figure, a comparable experiment in which the cells were at 37° during exposure yielded an aberration percentage of 12 at the 50-millijoule irradiance level. Surprisingly, the curves do not saturate to a constant aberration frequency. With increasing irradiances (time?), the aberration percentage drops. In this respect, the response of kidney cells is similar to several of the photomorphogenic responses of plants mediated by phytochrome; extended exposure to one band will simulate the effects of the other by virtue of the appreciable extinction of both forms of the chromoprotein in adjacent wavelengths.

The preceding experiment denigrates the likelihood

FIG. 73.—Several of the aberration types observed in pig kidney cells after an exposure to far-red light at 18° C at an irradiance of 75 millijoules cm^{-2} . The upper part of the figure shows chromatid and isochromatid breaks, interchanges, and fragments. The lower part of the figure shows a chromatid break, "mares-tails," and chromosome clumping.

that the far-red light acts via a macrothermal denaturation. First, the aberration yield increases as the temperature drops. In this respect, the kidney cells resemble the microspores of *Tradescantia*. With such microspores there is no effect of the light at temperatures above 20°, but potentiation becomes increasingly pronounced as the temperature is lowered.⁽²⁾ Second, the cytological response to far-red light is reversed by red light.⁽³⁾ Third, the monochrometers employed involve successive passage of light from a tungsten filament through infrared

FIG. 74.—The aberration yield in pig kidney cells at various times after exposure to far-red radiation.

reflecting glass, 10 cm of ferrous ammonium sulfate solution, and an interference filter of the Fabry-Pirot type, with blocking filters lowering the side band energies to less than 0.2% of the principal. Thus the intensities of wavelengths from 800 mμ to the infrared may be considered to be of relatively low order.

In Figure 72 the various types of aberrations observed were summed. These types appear to cover a cytological spectrum similar to those induced by ionizing radiation. The preponderant abnormality observed was the chromatid break, with a fairly high frequency of "stickiness," interchanges, and intrachanges. Several examples of the aberrations observed are illustrated in Figure 73.

Experiment III

An attempt was made to determine the time after irradiation at which the aberration frequency was at its maximum. Eighteen hours after subculture, kidney cells in Leighton tubes were exposed to 75 millijoules cm⁻² at 17°. They were then returned to the dark at 37°. Periodically tubes were taken and treated with Colcemide. Summed metaphase aberrancies scored for the various samples are shown in Figure 74. A maximum aberration frequency was observed for the cells sampled at 12 to 18 hours after irradiation. We suspect that the

time after irradiation at which the maximum aberration frequency occurs varies with the generation time and the stage in the cell cycle at which irradiation occurs.

Experiment IV

It has been shown elsewhere⁽⁴⁾ that subculture induces a partial phasing of the cell cycle. The responses described in Figures 72–74 are of cells subcultured at 37° for 18 hours and then irradiated. At what stage of the cell cycle did the irradiation occur? The extent of incorporation of tritiated thymidine introduced at various times after subculture was determined. From the results shown in Figure 75, it may be inferred that irradiation occurred at S, or the phase of DNA synthesis. To check this inference, we are determining the relative sensitivity to far-red radiation of the phases of the cell cycle. Preliminary experiments in which the cells are irradiated at various times after subculture indicate maxima of sensitivity corresponding to the periods of high rates of DNA synthesis.

FIG. 75.—The uptake of tritiated thymidine by nuclei of pig kidney cells at various times after subcultivation.

Experiment V

A hypothesis was suggested⁽¹⁾ proposing that the potentiating action of far-red light is caused by the sensitivity to light of metabolic energy transfer. We suggest that a similar hypothesis applies to the cytological sequelae in cells exposed only to far-red light. The experiment, described in Figure 76, measures the effect of far-red light on the esterification of inorganic phosphate by pig kidney cells. Cells were exposed to an irradiation protocol similar to those described in preceding experiments and assayed four hours after irradiation. The results indicate that exposure to far-red light causes a 20% reduction, significant at a >99% probability level, in the capacity of the cells to esterify *ortho*-phosphate.

We propose that maintenance of chromosome integrity is limited metabolically by the availability of ATP. Either a highly dynamic rupture and restitution

of chromatin bonds, or the biosynthesis of DNA, could be limited by competitive demand for high energy phosphate. Implicit in this speculation is the idea that any metabolic impairment leading to a decrease in energy availability, particularly during DNA synthesis, would increase the probability of chromosomal aberration. It is pertinent that other inhibitors of ATP formation, such as respiratory uncouplers, raise aberration frequencies⁽¹⁾ and increase ionizing-radiation lethality.⁽⁵⁾ Both ultraviolet and ionizing radiations cause neoplasia as well as chromosome aberrations. It would be of interest to know whether far-red light has a tumorigenic action as well.

REFERENCES

1. Gordon, S. A. Oxidative phosphorylation as a photomorphogenic control. *Quart. Rev. Biol.* **39**, 19-34 (1964).
2. Swanson, C. P., C. S. Rupert and H. T. Yost, Jr. Infrared absorption and temperature studies on the buds and chromosomes of *Tradescantia paludosa*. *Am. J. Botany* **40**, 557-565 (1953).
3. Moh, C. C. and R. B. Withrow. Nonionizing and radiant energy as an agent in altering the incidence of X-ray induced chromatid aberrations. II. Reversal of the far-red potentiating effect in *Vicia* by red radiant energy. *Radiation Res.* **10**, 13-19 (1959).
4. Stroud, A. N., A. M. Brues and B. Svoboda. DNA synthesis in mammalian kidney cells in tissue culture after single and periodic doses of irradiation. *Ann. N.Y. Acad. Sci.* **95**, 942-959 (1961).
5. Smith, W. W. and F. Smith. Effect of thyroid hormone on radiation lethality. *Am. J. Physiol.* **165**, 639-650 (1951).

Experimental Protocol

1. 10^5 cells, sub-cultured 18 hrs, 37° , darkness
2. 15 min, 17° , darkness
3. Far-red, 75 millijoules, 17° (5 min)
4. 4 hrs, 37° , darkness
5. $1 \mu\text{C } P^{32}$ introduced
6. 1 hr, 37° , darkness, Lindberg-Ernster fractionation

TIME INCUBATED WITH P^{32}	P_o CTS/MIN (CORR)	n
0'	41 ± 7	6
60' CONTROL	470 ± 14	5
60' Far-red	380 ± 19 ~ 20 % reduction	6

FIG. 76.—The effect of far-red light on the conversion of inorganic to organic (P_o) phosphate by pig kidney cells

THE GENETIC ANALYSIS OF RADIATION SENSITIVITY

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

PURPOSE AND METHODS

The genetic basis for variation in radiation sensitivity has been studied for two primary reasons—to evaluate the extent to which this genetic variable may exist in populations; and to examine the genetic mechanisms controlling a quantitative physiological genetic system. The genetic methods are standard and include the comparison of different inbred strains of mice, selected hybrid and backcross generations. The study has led to the development of a new statistical method for determining genetic recombination between a quantitative and qualitative trait.⁽¹⁾

The biological end points have usually been some

measure of survival: the 30-day LD_{50} , mean after survival (MAS) from the time of radiation exposure, age specific mortality rates, and major pathology at the time of death. The animals have been subjected to single whole-body doses of X- and γ radiation and daily doses of γ radiation for the duration of life. Most exposures were made or initiated when the mice were between 60 and 100 days of age.

PROGRESS REPORT

Data and analysis of the genetic variation in the acute lethal response to single whole-body X-radiation and the analysis of life shortening among the survivors

of the acute syndrome have been reported.⁽²⁻⁴⁾ These data indicated that radiation resistance is a dominant trait, possibly exhibiting over-dominance. In addition, a linkage relation was detected between the albino locus and radiosensitivity.

A recurrent backcross series was initiated from the F₁ hybrids of the BALB/c:C57BL/6 cross. The radio-sensitive BALB/c parent (P₁) was the recurrent parent and has the coat color genotype AAbbcc, agouti (A),

TABLE 14
LD₅₀ VALUES FOR BACKCROSS GENERATIONS

Generation	% Non-P ₁	LD ₅₀ ± SE	Family	LD ₅₀ ± SE
BC _I	25.0	635 ± 7	Albino Nonalbino	625 ± 8 643 ± 3
BC _{II}	12.5	596 ± 17	Albino Nonalbino	572 ± 16 622 ± 6
BC _{III}	6.25	561 ± 8	Albino Nonalbino	555 ± 13 581 ± 23
BC _{IV}	3.125	557 ± 4	Albino Nonalbino	547 ± 7 577 ± 1
BC _V	1.562	548 ± 7	Albino Nonalbino	545 ± 4 559 ± 5
BC _{VI}	0.781	564 ± 4	Albino Nonalbino	556 ± 6 575 ± 10
P ₁	0	559 ± 9		
F ₁	50.0	716 ± 7		

brown (b), and albino (c). The C57BL parent (P₂) is radiation resistant and has the genotype aaBBCC, non-agouti, black, nonalbino. The F₁ is heterozygous for the three loci, AaBbCc, while the first backcross generation, BC_I, segregates albino:nonalbino in a 1:1 ratio. Subsequent generations were produced within two backcross family lines, albino and brown agouti. The results of this series are given in Table 14.

The LD₅₀ values for the backcross generations move steadily toward the recurrent parent consistent with expectation for backcrosses involving additive genetic factors—the genetic difference between P₁ and F₁ should be reduced at the rate of $(\frac{1}{2})^n$ where n is backcross generation number. The data permit the genetic hypothesis that radioresistance in this test system is attributable to a number of independent gene pairs that act additively, while within the allelic pair, resistance is dominant. The observation that the F₁ was higher than the high parent can be attributed to transgressive inheritance; P₁ apparently carries one or more genes for resistance that are not common to P₂. This produces an F₁ value above P₂ that is not due to over-dominance.

The analysis of linkage between the albino gene and

radiosensitivity was accomplished in the recurrent backcross series by comparison of the data from the albino with that from the nonalbino family.⁽¹⁾ Recombination was estimated to be about 21%, and about 30% of the total difference between the F₁ and P₁ was estimated to be controlled by the linked gene or block of genes.

The determination of recombination frequency then permitted certain predictions of the LD₅₀ values for the original F₂ data and for data on the cross between the BC_I and the F₁. The predicted and observed values are given in Table 15. Excellent agreement between observed and predicted was noted in all cases, but the magnitude of the differences among the several families is small so that these tests are not particularly critical. The important feature is the continued consistency of all the data with the derived explanation of the genetic behavior of the trait "radiosensitivity"—interallelic additivity with intraallelic dominance of resistance and a linkage in coupling between the albino locus and sensitivity.

Analysis of the survival statistics (Table 16) of inbred strains A/Jax, BALB/c, C3H₁, C57BL/6 and the BALB:C57BL F₁ and F₂ generations under duration-of-life gamma radiation has provided an opportunity to examine the genetic variation in viability over the whole lifetime. Statistically significant strain differences in MAS exist at all dose levels, while sex differences are

TABLE 15
OBSERVED VERSUS PREDICTED LD₅₀ VALUES FOR SEGREGATING GENERATIONS DERIVED FROM
BALB/c:C57BL/6 CROSS

Generation or Cross	Family	Coat Color Segregant	Observed LD ₅₀	Predicted LD ₅₀
F ₂		Albino Nonalbino	602 ± 9 639 ± 8	601 626
BC _I × F ₁	Albino	Albino	632 ± 15	632
	Nonalbino	Nonalbino	654 ± 1	656
		Albino	641 ± 2	636
		Nonalbino	656 ± 5	661

significant only at 0, 74, 97, and 125 R per day. The life expectancy of a given strain at a given dose is generally a function of the normal life expectancy of the strain. It has, in fact, been demonstrated that the ln MAS vs arithmetic daily dose relationship over the range 12 R to 56 R/day is linear and is described by the slope—0.038 days/R/day.^(6, 6) No significant variation attributable to sex or strain could be demonstrated for this life shortening parameter. It describes the life shortening effect of daily radiation for all mouse strains and hybrids so far tested and is tentatively assumed to be a species constant. Genetic differences are seen among the 0 R

intercepts or among the MAS values at any other selected abscissal point.

High, between-strain correlations are demonstrated between the young adult single dose LD₅₀ value and the MAS values at each of the dose levels. The correlation lies between +0.92 and +0.95 for the doses from 12 R to 56 R/day, while at all other levels it lies between +0.79 and +0.85. These data further support the thesis put forth several years ago⁽⁴⁾ that the young adult LD₅₀ value could be accepted as an accurate indicator of the general viability and after expectation of life of the animals tested.

ceptible to unpredictable environmental interactions.⁽⁶⁾

Heritability (h^2) of a quantitative trait is a measure of the portion of the total phenotypic variance attributable to only additive genetic factors. It was estimated from these data by two standard procedures:⁽⁷⁾ the comparison of inbred strains, and the comparison of inbred parental with genetically segregating generations. The results are generally comparable and indicate an average value for h^2 of 0.55 or greater. The values vary among dose levels from 0.06 to 0.88, and the two methods of estimation are equivocal on the point of any existing correlation between h^2 and MAS. The values de-

TABLE 16

MEAN AFTER SURVIVAL UNDER DURATION-OF-LIFE CO⁶⁰ GAMMA IRRADIATION STARTED AT 100 DAYS OF AGE

Strain	Sex	Dose, R/Day											
		0	5	12	24	32	43	56	74	97	125	170	220
A/Jax.....	M	458		358	219	174	96	67	42	36	26	18	16
	F	487		329	231	173	92	64	43	32	22	18	17
BALB/c.....	M	387	349	325	180	147	91	53	47	33	20	17	16
	F	453	461	319	211	153	85	56	43	27	21	18	16
C3H ₁ /He.....	M	399		382	256	192	131	83	51	36	22	18	17
	F	424		381	266	177	119	86	49	31	22	19	16
C57BL/6.....	M	576	615	440	251	205	120	84	54	40	32	24	18
	F	584	522	432	251	198	124	80	53	37	31	23	17
(BALB:C57BL)F ₁	M	759		425	294	229	160	101	65	44	34	22	18
	F	782		433	284	217	152	100	57	43	29	21	19
(BALB:C57BL)F ₂	M	548		407	259	203	129	86	56	40	31	20	17
	F	561		408	269	196	134	81	51	36	25	20	18
Sample size per sex and strain.....		50-72	27-33	30-42	33-42	30-42	33-42	24-30	27-30	27-30	24	18	12

The previously noted variance analyses of the MAS values were executed in two ways. One, the observed arithmetic data on days of survival were analyzed, and two, the data were transformed by the function (MAS)^{-0.4} to eliminate the positive correlation between mean and variance that characterizes survival data. The transform function is entirely empirical and is derived from the observed nonlinear relationship between the MAS values and the appropriate within-cage variances.

This effort to stabilize the variances is important to the genetic analysis. Estimation of heritability, for example, can not be done with the observed variances since genetic and environmental variance components are confounded by the positive metrical bias. The variance stabilizing transform did succeed in eliminating most of the bias. It was least successful for control values, which is not surprising in view of our general observations that control animals appear most sus-

rived from the variance among inbred strains show a cyclic behavior that is inversely related to the impulse lethality function,⁽⁵⁾ while those derived from the F₂ data show only random fluctuation.

CONCLUSIONS

The analysis of the genetic basis of the quantitative trait "radiosensitivity" is now fairly complete. The trait is controlled by a number of gene pairs that act additively although members of the allelic pairs exhibit dominance for resistance over sensitivity. This analysis is based upon the acute response of young adults to single dose whole-body exposure. The derived LD₅₀ values are positively correlated to survival under daily γ radiation at all levels tested between 0 and 220 R/day. Resistance to acute radiation injury is thus an indicator of greater general viability. The latter trait has an average heritability greater than 0.5.

REFERENCES

1. Grahm, D., K. F. Hamilton and W. P. Leslie. Analysis of linkage between qualitative and quantitative traits in mice. *Genetics* **47**, 955-956 (1962).
2. Grahm, D. and K. F. Hamilton. Genetic variation in the acute lethal response of four inbred mouse strains to whole-body X-irradiation. *Genetics* **42**, 189-198 (1957).
3. Grahm, D. Acute radiation response of mice from a cross between radiosensitive and radioresistant strains. *Genetics* **43**, 835-843 (1958).
4. Grahm, D. Genetic control of physiological processes: The genetics of radiation toxicity in animals. *A Symposium on Radioisotopes in the Biosphere*. Ed. R. S. Caldecott and L. A. Snyder. University of Minnesota Press, Minneapolis, 1959. Ch. 14, pp. 181-200.
5. Sacher, G. A. and D. Grahm. Survival of mice under duration-of-life exposure to gamma rays. I. the dosage-survival relation and the lethality function. *J. Natl. Cancer Inst.* **32**, 277-321 (1964).
6. Grahm, D., G. A. Sacher and K. F. Hamilton. A uniformity of life shortening among genetically different mice under daily gamma radiation. *Radiation Res.* **19**, 239 (1963).
7. Falconer, D. S. *Introduction to Quantitative Genetics*. The Ronald Press Co., New York, 1960.

MUTATION IN CONTINUOUS CULTURES

Herbert E. Kubitschek

PURPOSE AND METHODS

Our purpose in the study of mutation in continuous cultures of bacteria is to delineate the sequence of events occurring during the mutational process for at least one mutational system. For this purpose, we assume that it is necessary to use low doses of mutagen in order to minimize cell death and physiological perturbations. Almost all of our work has concerned the mutation from cell sensitivity to the resistance to the lytic bacteriophage T5. This system has several advantages. Most studies of mutation have concerned back mutation to prototrophy; mutation to phage resistance is a forward mutation. A more important feature is that T5-resistant cells are not under selection in chemostat cultures, so that measurements of mutation frequencies give direct measurements of mutation rates without the need for correction for selection factors.

In this study it is necessary to determine the kinetics of induction and of expression of mutation in cultures exposed to various mutagens. Cells that are phenotypically mutant are determined simply by exposing culture samples to bacteriophage T5 upon nutrient agar plates; sensitive cells absorb the bacteriophage and are lysed, while cells that have mutated to resistance survive and give rise to visible colonies. We also determine the concentration of latent mutants, those cells in the culture that are not yet phenotypically mutant but in which the mutational process has been initiated. Latent mutant concentrations are measured by spreading culture samples upon plates and incubating these plates for 3 hours at 37° C before spraying them with bacteriophage. During this period microcolonies of about 100 cells are formed from each cell present on a plate, allowing a mutant gene in an original cell to give rise to one or more phenotypic mutants among its progeny.

PROGRESS REPORT

Most of our recent work has centered on photodynamic induction of mutation with acridine orange and a short exposure to visible light since it is possible in this way to obtain a significant mutant yield in the absence of noticeable cell death and with, at most, a transitory change in rate of cell division. In slowly growing cultures, in which cells are unlikely to be multinucleate, we found that latent mutant concentrations appear to reach their maximum value immediately after exposure of the culture to light. Phenotypic expression, however, can take many generations; few or no mutations are expressed during the first cell division, but thereafter, latent mutant concentrations appear to diminish approximately exponentially with a loss of about 50% each cell division. The concentration of phenotypically expressed mutants ultimately becomes indistinguishable from the initial concentration of latent mutants; that is, every latent mutant appears to give rise to completely mutant progeny lines.

CONCLUSIONS

These results require that few, if any, mutating cells undergo a segregational division, and therefore, either that 1) both strands of DNA are mutated, or 2) if only one of the DNA strands is mutated, then this strand must specify the coding sequence of both daughter strands during replication of DNA. The latter explanation is preferable because it permits a model of mutation that predicts the observed exponential kinetics of phenotypic expression. Thus our results suggest that the Watson-Crick model for replication of DNA requires modification to the extent that both newly forming daughter strands of DNA are specified by a single parental strand.

AUTOMATIC CLASSIFICATION OF CHROMOSOMES

Agnes N. Stroud, James W. Butler,* Margaret Butler,* and Nancy A. Munro

PURPOSE AND METHODS

With the growing interest in radiation-induced chromosome aberrations and in the genetic origin of congenital malformations, or "inborn errors" recently found in man due to chromosomal translocations, nondisjunctions, or random separations, a faster procedure for recognizing chromosomes was inevitable so as to process hundreds of cells of one individual to obtain reliable statistical data. As a result it became necessary to make use of the rapid information processing capabilities of digital computers and a scanning system to reduce the time expended in the tedious process of karyotyping.

In collaboration with the Applied Mathematics Division, chromosomes of a metaphase spread are measured and digitized on 35-mm film with the CHLOE machine, a digitally controlled precision cathode ray tube (CRT) film scanner, an IBM 704 program, and the computer GEORGE.

Our present experiments on the effects of ionizing radiations on mammalian tissue are particularly concerned with the induction of chromosome changes by radiation. Some of these changes are easily detectable by eye but may involve only a minute exchange of chromosome material which could only be reliably detected by a scanning device such as CHLOE. For this system the biological method employed in obtaining cells which exhibit their chromosomes for morphologic and microscopic examination is known as the tissue culture method. Once the cells are in mitotic division, they are arrested during the metaphase stage with a drug in order to increase the proportion of cells in this stage of division and to aid in spreading of the chromosomes. The cells are then briefly exposed to a hypotonic physiological saline solution to increase the cell volume so that the chromosomes become distinct entities with a random arrangement. They are later killed with a fixing agent, spread on a microscopic slide and permitted to air dry to ensure additional separation of the chromosomes within the cell. For photography the chromosomes are stained with a dye. The chromosomes, which are now randomly arranged in a metaphase spread, are photographed through a photomicrographic camera on 35-mm film, resulting in a 1000-fold magnification of the individual chromosome.

The measurement function and part of the data processing are performed by the CHLOE film measuring system. It serves as a means of digitizing photographic information and performing necessary compu-

tations with the numerical data thus obtained. The system consists of an optical scanner and a cathode ray tube (CRT). A spot of light from the CRT is projected onto the film, and the transmitter light is registered by a photomultiplier by means of which a decision is made regarding the density. A rectangular area on the film is scanned and the extent of the area in both directions is determined by the computer program. The spot of light is stationary for 1 microsecond, is blanked out for 3 microseconds and reappears in the adjacent location for 1 microsecond. When a change in the intensity of the transmitted light from one point to another is detected by the photomultiplier, the contents of the counter are sent into the computer memory as the coordinates of the point. The local density of the photograph, quantized into 8 levels, is also transmitted to the computer.

This system at the moment requires 3 digital computers, the ASI-210, IBM 704, and Argonne-built computer GEORGE, and four different programming languages. Eventually it will be directly connected to the 3600 computer. Measurement and shape construction are controlled by the ASI-210 computer which forms part of the CHLOE system; the remaining operation, with the exception of picture reconstitution, are performed by the IBM 704 program. Reconstitution of the pictures from the digital information is done with the Argonne-built computer GEORGE, which is equipped with a cathode-ray tube output device. Input for this step is a magnetic tape which is written out by the IBM 704 at an early stage in the processing of the data.

After the individual chromosomes are recognized by the computer programs, each one is characterized by a set of seven numbers called Euclidean moment invariants, which are independent of the position of the chromosome on the picture and its orientation. Each chromosome is thereby representable as a point in a seven dimensional measurement space and a metric is then defined in such a way that chromosomes with nearby representative points will tend to be similar in shape.

The objective of the IBM 704 computer program is to pair each chromosome with its corresponding one on the basis of shape similarity and thus detect aberrant chromosomes caused by radiation damage or other abnormal conditions.

PROGRESS REPORT

The CHLOE program which has been under way for one year is now producing statistical results. The efficiency of pairing all the chromosomes with their

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FIG. 77.—The reconstituted metaphase spread direct from the CRT output device with connecting lines drawn by the computer

FIG. 78.—The original metaphase spread with 36 chromosomes before automatic classification

homologues has been as high as 90% in some cases. Since there are some pairs which fall into groups of almost the same size and shape, CHLOE has paired homologous chromosomes 50% better than an individual. The process can be improved by further refinement of the program and the remedying of certain defects which still remain in the system.

The final output of the process is in the form of reconstituted pictures, printed output, and punched cards. A typical reconstituted picture direct from the CRT output device with connecting lines drawn by the computer program is shown in Figure 77, illustrating the pairing done by the computer program. The original metaphase spread with 36 chromosomes is shown in Figure 78. These

figures indicate that a rather high degree of success has been achieved in the computer pairing operation.

CONCLUSIONS

A new technique for classifying somatic chromosomes according to their homologous pairs, with the exception of the unpaired male sex chromosomes, has been developed which involves digital computer techniques.

It is now becoming more probable that the tedious job of pairing chromosomes and detecting chromosome aberrations will be taken over by computers, which can scan and produce information on randomly spread chromosomes of a single cell about 2500 times as fast as a technician.

DISTRIBUTION PROPERTIES OF SEX RATIO IN MICE AND THE FREQUENCY OF SPONTANEOUS SEX-LINKED MUTATIONS

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

PURPOSE AND METHODS

The object of this study is to estimate in a constant environment the spontaneous mutation rate for sex-linked lethal genes in the mouse. To our knowledge, this has not been done previously. In addition, tests of significance for genetic and environmental variation in sex ratio are conducted.

Data on the sex ratio at birth for 1630 single pair matings set up for the routine production of experimental animals from 6 inbred and 9 hybrid strains and several backcrosses were available. The succession of litters from a mating pair was considered a random sample for the aggregate or population of litters. From the random sample of litters consisting of N individuals, M of which are males, one may compute a sex ratio by the fraction M/N . However, due to strain differences, the population is heterogeneous, with each strain having a characteristic sex ratio. The mating pairs were, therefore, classified according to strain and the strain sex ratios were computed.

In the preliminary analysis the deviation χ^2 with one degree of freedom was used to test the observed sex ratio proportion for each mating pair against the strain expectations. The mating pairs within a strain were subsequently classified in a $(2 \times n)$ -fold table and the χ^2 test of homogeneity was applied to litters within mating and matings within strain. The results obtained indicated that a more exact test was needed. Exact evaluation of the binomial probabilities of observed distributions (and those less probable) was computed, based on the observed proportion of males.

PROGRESS REPORT

If the matings differ only by chance, the theory of random sampling leads us to expect a certain measure of departure from the expected ratio in finite samples. If the matings differ, not only by chance but by a tendency on the part of some parents to produce males or females, then the frequency distribution of sex ratio should show an excess of unequally divided families. The frequency distribution of the proportion of males is shown in Table 17. Preliminary statistical analysis and graphical representation indicate a slight tendency for skewness and kurtosis.

The presence of a spontaneous sex-linked recessive lethal gene in a female will decrease the number of males produced by the mating pair and the sex ratio will approach 0.33 ($2\text{♀} : 1\text{♂}$) if there exist no correcting or compensating factors. Unfortunately, females with the XO genotype will also produce litters showing a 0.33 sex ratio, and in the present data these females cannot be distinguished from XX females carrying a recessive lethal. As yet, there are no genetic factors except sex-limited lethals to explain a significant deficiency of females. If there are nongenetic factors effecting differential embryonic mortality, they are confounded with estimates based on a deficiency of either sex when secondary sex ratio is used.

χ^2 analysis of the data showed that the total number of matings with a deficiency of male offspring is greater than the total number of matings with a deficiency of females. This was considered evidence for the presence of sex-linked lethal genes. Unfortunately, the total ran-

dom deviation was depressed and further investigation of the distribution was required. The results showed that the expected inflation of the χ^2 value from the presence of heterogeneity was not always large enough to be detected.

Binomial analyses of the data showed significant deviation in excess of random expectations, but the number of matings showing a deficiency of males was less than the number of matings showing a deficiency of females. This excess deficiency of females was due mainly to one strain, the C57BL/6.

these breeding records, one sex-linked lethal gene did occur but it was first recorded in a male, undoubtedly of the XXY genotype. Consequently, it was not a part of the population under scrutiny here (the reproducing females) and, therefore, is not included in the analysis. The mutation was "Tortoise" (To) and is characterized by a mottled coat color in the carrier female and is lethal in the male, apparently before implantation. The gene was first reported by Fraser et al.⁽²⁾ and its mottled expression in the female is characteristic under the hypothesis of random inactivation of the mammalian sex

TABLE 17
FREQUENCY DISTRIBUTION OF PROPORTION OF MALES

STRAIN	NUMBER OF MATING PAIRS	SEX RATIO	PROPORTION OF MALES BORN									
			-0.10	0.11-0.20	0.21-0.30	0.31-0.40	0.41-0.50	0.51-0.60	0.61-0.70	0.71-0.80	0.81-0.90	0.91-
A/He.....	179	0.485	0.006	0.011	0.039	0.156	0.408	0.257	0.089	0.022	0	0.011
A/Jax.....	197	0.507	0	0.015	0.030	0.117	0.325	0.360	0.117	0.036	0.005	0
BALB/c.....	223	0.480	0.009	0.018	0.054	0.161	0.399	0.251	0.085	0.018	0	0.004
C3H ₁ /He.....	183	0.509	0.005	0.005	0.005	0.120	0.388	0.355	0.081	0.038	0	0
C57BL/6.....	216	0.519	0	0	0	0.069	0.370	0.394	0.134	0.019	0.014	0
BCF ₁	151	0.480	0	0	0.007	0.126	0.477	0.318	0.046	0.020	0	0.007
(C57BL/6×BALB/c)												

The data gave no evidence of an effect of litter size, age of dam, or parity on the sex ratio. A more precise interpretation will be possible when a partial regression analysis is completed.

Applying the method outlined by Stevens⁽¹⁾ the pooled spontaneous mutation rate for sex-linked lethal genes is estimated as 1×10^{-3} per X-chromosome per generation. It is important to recognize that this is a maximum estimate, since it includes females with the XO genotype which cannot be identified or eliminated without the use of marker stocks.

During the 10- to 12-year period of accumulation of

chromosome.⁽³⁾ The occurrence of sex-linked mutations in genetically abnormal males is not unknown, but it offers no solution to the estimation of spontaneous mutation rates since most lethals may have to be detected entirely by statistical measures of disturbed sex ratios rather than by visible phenotypic effects.

REFERENCES

1. Stevens, W. L. Accuracy of Mutation Rates. *J. Genetics* **43**, 301-307 (1942).
2. Fraser, A. S., S. Soley and C. C. Spicer. "Mottled," a sex modified lethal in the house mouse. *J. Genetics* **51**, 217-221 (1953).
3. Lyon, M. F. Sex chromatin and gene action in the mammalian X-chromosome. *Am. J. Human Genetics* **14**, 135-148 (1962).

METABOLISM OF FISSION PRODUCTS

TOXICITY AND METABOLISM OF RADIONUCLIDES. STATEMENT OF PROGRAM OBJECTIVES

William P. Norris

PURPOSE AND METHODS

The objective of this program is the systematic study of the effects of selected fission products administered singly or as well-defined, simple mixtures. Radionuclides selected for study were chosen as being representative of one of the following types: 1) "biologically soluble" radioisotopes which will irradiate an entire animal more or less uniformly; 2) "biologically insoluble" radioisotopes which are preferentially taken up by the phagocytic cells of the reticuloendothelial system and, to some considerable extent, by bone; 3) "bone seekers" which are almost entirely restricted to the skeleton; 4) iodine, which by virtue of its chemistry is preferentially deposited in the thyroid gland.

Representatives of Classes 1 and 2— Cs^{137} and Ce^{144} , respectively—have been given intravenously in single doses to pure-bred beagle dogs. The results have served to define the picture of acute lethality of these radionuclides in dogs. These isotopes have also been administered in doses less than those found acutely lethal for the demonstration of their late effects.

Since Class 1 radionuclides do deliver radiation dosages in a reasonably uniform manner to the animal body, it is of interest to simulate this exposure using

whole-body irradiation from an external source. This requires that the dosage rate be decremented in a fashion similar to the biological excretion of the isotope. The first experimental phase of this comparison, using Co^{60} γ rays, is currently nearing completion.

A tracer study of the uptake and retention of I^{131} and I^{125} in the thyroid glands of beagles is essentially complete. This was considered a necessary preliminary to the administration of I^{131} in doses sufficient to produce visible signs of radiation damage.

It is clear that a study of the effects of irradiation from sources composed of mixtures taken from Classes 1–4 and external whole-body irradiation will do much to elucidate the hazards of mixed fission products. Such an experimental study will begin as soon as sufficient information is compiled using members of these classes singly.

It is important to view these animals in sufficient detail to obtain a comprehensive picture of alterations in their biochemistry, physiology, clinical status, radioactive content, pathological changes, and radiation dosimetry. To the extent permitted by the present staff, these observations are being made, and the results will be included in the following reports.

CESIUM-137—TOXICITY AND RETENTION IN DOGS OF DIFFERENT AGES

William P. Norris, Louise S. Lombard, Calvin M. Poole, Carl E. Rehfeld, and Norbert D. Kretz*

PURPOSE AND METHODS

A total of 73 beagle dogs was given Cs^{137} intravenously in doses near that which is lethal within 30 days after injection. The pertinent information concerning each experimental animal and its survival following treatment with Cs^{137} is contained in Table 18. One will

note that these animals fall into three age groups at the time of radionuclide administration: 5 months, 13 months, and 4–5 years.

PROGRESS REPORT

The $\text{LD}_{50/30}$ for 13-month-old dogs was determined experimentally at the outset and found to be approximately 3.5 mCi/kg of body weight. The injected ani-

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TABLE 18
SURVIVAL OF BEAGLES FOLLOWING A SINGLE INTRAVENOUS DOSE OF Cs¹³⁷

Dog No.	SEX	DATE INJECTED	AGE AT INJECTION, DAYS	AMOUNT		DAYS TO DEATH	SURVIVAL* AT 9-22-64, DAYS	CAUSE OF DEATH	
				mCi	mCi/Kg				
5-Month-Old Dogs									
470.....	M	10-23-62	151	25.22	3.85		700	Bone marrow aplasia	
398.....	M	4-17-62	151	32.27	3.79	27			
473.....	F	10-23-62	151	21.22	3.79		700		
492.....	F	10-31-62	148	22.10	3.76		692		
471.....	M	10-23-62	151	21.91	3.76		700		
487.....	M	10-31-62	149	25.79	3.70	39		Bone marrow aplasia	
401.....	F	4-17-62	151	29.40	3.68	27		Bone marrow aplasia	
397.....	M	4-17-62	151	35.29	3.67		889		
404.....	F	4-17-62	142	30.52	3.65		889		
491.....	F	10-31-62	148	20.09	3.65		692		
472.....	F	10-23-62	151	17.19	3.65		700		
485.....	M	10-31-62	149	27.50	3.64		692		
474.....	F	10-23-62	151	23.80	3.60		700		
486.....	M	10-31-62	149	24.57	3.59		692		
399.....	M	4-17-62	151	30.26	3.24		889		
13-Month-Old Dogs									
417.....	M	2- 5-63	388	45.68	4.31	27			Bone marrow aplasia
418.....	M	2- 5-63	388	44.31	4.28	26		Bone marrow aplasia	
416.....	F	2- 5-63	388	44.16	4.25	24		Bone marrow aplasia	
419.....	M	2- 5-63	388	36.61	4.14	27		Bone marrow aplasia	
241.....	F	1-18-62	417	26.48	3.69	31		Bone marrow aplasia	
237.....	F	1-18-62	417	31.91	3.59	28		Bone marrow aplasia	
438.....	M	5- 1-63	391	37.83	3.54		510		
437.....	M	5- 1-63	391	33.57	3.46		510		
392.....	F	12- 5-62	395	26.77	3.45		657		
393.....	F	12- 5-62	395	23.85	3.41		657		
435.....	M	5- 1-63	391	35.01	3.40		510		
436.....	M	5- 1-63	391	35.34	3.40		510		
239.....	M	1-18-62	417	34.44	3.36		978		
391.....	F	12- 5-62	395	28.03	3.34		657		
243.....	M	1-18-62	417	40.22	3.33	35			Bone marrow aplasia
390.....	F	12- 5-62	395	29.27	3.31		657		
313.....	F	11- 6-62	561	27.73	3.18	7		Euthanized for tissue study	
366.....	F	11- 6-62	399	24.26	3.17		686		
316.....	F	11- 6-62	561	28.34	3.14	14			Euthanized for tissue study
369.....	M	11- 6-62	399	24.52	3.10	28			Bone marrow aplasia
367.....	M	11- 6-62	399	25.91	3.09	36			Bone marrow aplasia
450.....	M	5-21-63	427	25.00	2.84		530		
428.....	F	5-21-63	415	21.49	2.72		530		
441.....	F	5-21-63	413	27.31	2.70	52			Bone marrow aplasia
451.....	M	5-21-63	427	29.05	2.70		530		
452.....	M	5-21-63	427	27.91	2.68		530		
430.....	F	5-21-63	415	26.55	2.65		530		
449.....	M	5-21-63	418	33.20	2.65	38		Euthanized for tissue study	
246.....	F	9-19-61	398	22.52	2.43		1099	(Tumor of left ovary removed)	
258.....	F	9-19-61	404	24.66	2.32		1099		
256.....	F	9-19-61	404	18.50	2.27		1099		
257.....	F	9-19-61	404	20.20	2.23		1099		
247.....	F	9-19-61	398	21.86	2.16		1099		
55.....	M	4-11-61	397	17.17	2.11		1260	Accidental death	
53.....	M	4-11-61	415	22.62	2.11	0			
52.....	M	4-11-61	415	17.56	2.11	2			

* Living at the time of reporting except as otherwise noted.

TABLE 18—Continued

Dog No.	SEX	DATE INJECTED	AGE AT INJECTION, DAYS	AMOUNT		DAYS TO DEATH	SURVIVAL* AT 9-22-64, DAYS	CAUSE OF DEATH
				mCi	mCi/Kg			
13-Month-Old Dogs—Continued								
54.....	M	4-11-61	397	17.05	1.98		1260	
81.....	M	4-11-61	397	15.52	1.85		1260	
444.....	M	5-21-63	413	18.52	1.74		530	
443.....	M	5-21-63	413	18.09	1.73		530	
439.....	M	5-21-63	415	17.59	1.70		530	
453.....	F	5-21-63	435	18.42	1.66		530	
454.....	M	5-21-63	435	16.33	1.65		530	
Older Dogs								
46.....	F	9-25-62	1387	36.65	3.74	21		Bone marrow aplasia
69.....	M	9-25-62	1662	42.45	3.71	24		Bone marrow aplasia
72.....	M	9-25-62	1662	34.60	3.70	22		Bone marrow aplasia
71.....	M	9-25-62	1662	30.49	3.70	22		Bone marrow aplasia
48.....	F	9-25-62	1509	33.60	3.64	20		Bone marrow aplasia
65.....	M	11-20-62	2060	24.43	3.40	28		Bone marrow aplasia
60.....	F	11-20-62	2066	23.11	3.39	24		Bone marrow aplasia
63.....	F	11-20-62	2060	25.23	3.32	26		Bone marrow aplasia
64.....	M	11-20-62	2060	19.75	3.29	29		Bone marrow aplasia
80.....	M	11-20-62	2060	28.16	3.27	24		Bone marrow aplasia
107.....	M	2-16-61	ca. 1023	26.32	1.99	64		Bone marrow aplasia
121.....	M	2-16-61	ca. 1023	28.09	2.04	27		Bone marrow aplasia
124.....	M	{ 2-16-61 10- 2-61	ca. 870	20.40 16.15	1.58 1.04		1314	
114.....	M	{ 10-31-60 8-14-61 4-10-62	ca. 731	7.80 10.76 11.63	0.93 1.01 1.13		1422	
128.....	M	{ 10-31-60 8-14-61 4-10-62	ca. 762	11.75 11.89 12.83	1.08 0.99 1.01		1422	

mals are ordered in Table 18 according to the measured amounts (in mCi/kg body weight) they actually received. By inspection of Table 18, it is seen that there were no survivors at dose levels of 3.59 mCi/kg and above. Of 13 dogs given Cs¹³⁷ at doses between 3.00 and 3.54 mCi/kg, ten survived. Two animals died shortly after receiving dosages of 2.7 mCi/kg.

Of 14 5-month-old beagles which received Cs¹³⁷ in dosages between 3.59 and 3.85 mCi/kg, only 3 died. A comparison of the mortality data from the 5-month- and the 13-month-old groups suggests that Cs¹³⁷ is more damaging to the older group; however, the distribution of actual dosages is such as to make this result equivocal.

In the older (4-5 years) dogs, however, the results clearly demonstrate that Cs¹³⁷ is more damaging than in either of the two younger groups. Here animals succumbed to doses of Cs¹³⁷ as low as 1.99 mCi/kg. There were no survivors out of 10 animals given dosages of Cs¹³⁷ between 3.29 and 3.75 mCi/kg. In addition these

animals died at an average of 24 days after injection—significantly earlier than in the two groups of younger dogs.

As a general rule, all animals which died did so at around 30 days following Cs¹³⁷ administration. This is a time after initiation of acute radiation at which damage to the hematopoietic system is most clearly evident. This was verified by hematological studies of the peripheral blood. Nearly all animals which survived this period show strong indications of hematopoietic recovery starting at 30-35 days.

With two exceptions, all dogs which survived 39 days after injection are still alive. At this date the time interval since injection ranges from 510 to 1260 days. Some of the surviving males were ejaculated recently, and none produced sperm. Their testicles are smaller than normal, and they are presumed to be sterile. Only one other suggestion of a late effect has, so far, been observed. One of these survivors developed a malignant

tumor of the left ovary at around 1000 days. The tumor was removed surgically and the dog still survives.

The reason for the apparent age-dependence in the radiosensitivity of these dogs to Cs^{137} becomes evident from the whole-body measurements of cesium retention. Except for the first day or two after injection, when retention decreased more rapidly, the retention of Cs^{137} was closely described by a single exponential term. During the first 1–2 days retention decreased by 10–15% of the injected dose in animals of all ages. This period is presumed to reflect the time required for cesium to equilibrate in tissue.

The biological half-period of Cs^{137} was estimated

TABLE 19
BIOLOGICAL HALF-TIMES ($\text{BT}_{1/2}$) OF Cs^{137}
AND INTEGRATED RADIATION DOS-
AGES FROM INJECTED Cs^{137} TO DOGS
WHICH FAILED TO SURVIVE

Dog No.	$\text{BT}_{1/2}$ Days	Days to Death	Integrated Dose to Death, Rad
5-Month-old Dogs			
398.....	19	27	875
487.....	18	39	816
401.....	20	27	869
13-Month-old Dogs			
417.....	24	27	1124
418.....	23	26	1120
416.....	26	24	983
419.....	19	27	1069
241.....	18	31	951
237.....	18	28	858
243.....	19	35	953
369.....	23	28	813
367.....	23	36	927
4- to 5-Year-old Dogs			
46.....	47	21	771
69.....	44	24	970
72.....	35	22	898
71.....	26	22	839
65.....	34	28	715
60.....	28	24	769
63.....	39	26	887
64.....	27	29	882
80.....	40	24	832
Average.....			901

individually for most of these dogs from plots of retention values against time. In the 15 animals 5 months old at injection these ranged from 11.8 to 22.5 days and averaged 16.4 days. Similar values from 27 13-month-old dogs ranged from 15 to 26 days and averaged 20 days. In 10 dogs 4 to 5 years old at injection, the range of biological half-times was 26–50 days with an average of 37 days.

Thus cesium is excreted more slowly by older dogs. This results in an increased radiation dosage above that received by younger animals—the factor in integrated dosages being the ratio of the observed biological half-times. It is possible that the above result is related to differential biological damage in the three age groups which might, in turn, have affected the retention of Cs^{137} . This point will be checked using tracer doses of Cs^{137} .

It is more probable, however, that Cs^{137} retention in the dog is the age-dependent factor responsible for the mortality picture reported here. There have been no reports of a marked age dependence in radiosensitivity in mammals—except possibly at the two extremes of the age range. The integrated radiation dosage to death was computed for each animal which failed to survive. The only assumption in this computation was that Cs^{137} was uniformly distributed in the body of the dog. The approximate correctness of this assumption was verified by direct analyses of muscle and organs of the animals at the time of necropsy. The integrated radiation dosages are listed in Table 19, in which the animals are ordered by injection dosage in mCi/kg as they were in Table 18. In all three age groups the dosage ranged from 715 to 1124 rads, the average being 900 rads. The distribution of values indicates no major differences in radiosensitivity of these animals with age.

The irradiation pattern to which these animals were subjected is essentially one of continuous whole-body exposure with the daily dose rate being steadily decremented as Cs^{137} is excreted. At 3.5 mCi/kg the initial dose rate was approximately 50 rad/day. It seems reasonable to believe that the capability of the dog to recover from radiation-induced damage is reduced by each daily increment of dosage. Therefore, irradiation delivered at a dose rate which is decremented in proportion to this reduced capacity to recover should be most efficiently utilized in producing biological damage or death. This hypothesis is being checked by studies with whole-body γ irradiation using a Co^{60} source.

THE RESPONSE OF BEAGLE DOGS TO CONTINUOUS EXPOSURE TO Co^{60} γ RAYS

William P. Norris, Carl E. Rehfeld, Frank Williamson, Thomas E. Fritz, and Calvin M. Poole

PURPOSE AND METHODS

This study is a first step in an experimental comparison between the response of beagles to continuous whole-body irradiation from an external Co^{60} source and their response to injected Cs^{137} .

Thirteen beagle dogs, approximately 13 months old, were placed in the high-level γ -ray facility and exposed continuously to γ radiation from a Co^{60} source. Three other beagles housed under similar conditions in the near vicinity acted as controls. The daily exposures were estimated to be 300, 210, 147, 103, 72, and 50 R/day to the respective animals. The absorbed doses are being determined from measurements with LiF microdosimeters implanted subcutaneously in each experimental animal. The doses were delivered during 22 hours out of each 24-hour day. The first exposure day began at 10:00 on 9-18-64. The source was removed from 16:00 to 16:30 each day for observation of animals. It was also removed from 8:30 to 10:00 for observation and maintenance of the animals. The total exposure period lasted until 8:30 on 9-29-64—a total of 11 exposure days. All animals survived this 11-day period.

PROGRESS REPORT

The survival of these beagles to date is given in Table 20. Here it is shown that there is relatively little difference in the survival of dogs at the three highest doses. The cause of death was directly related to the decrease in the number of white cells in the peripheral blood. There was a fairly uniform decrease in circulating leukocytes with each successive day for 10 days. Six of the exposed dogs and 3 controls provided daily blood samples for enumeration of cellular elements. In the course of 10 days' exposure the drop in the numbers of circulating leukocytes occurred roughly in direct proportion to the size of the dose. However, the decrease was quite parallel in the two highest exposures and similarly in the third and fourth levels with the two lower doses being well separated from the high levels and from one another. At the end of 10 days there were only about 3% of the preexposure number of circulating leukocytes in the blood at the highest level of exposure in contrast to 50% at the lowest level.

Blood samples were obtained at selected intervals and cultured to check for the onset of bacterial septicemia. Tissues were also collected at necropsy for similar bacteriologic examination. In each case, septicemia preceded the death of the animals and probably contributed significantly as a cause of death.

Blood samples are also being collected at regular

intervals, and capillary whole blood clotting times (WBCT) as well as one-stage prothrombin times⁽¹⁾ are being determined. The WBCT of the dog receiving the 300 R was significantly lengthened the day preceding death, but the changes in the others have not been dramatic. In general, the prothrombin times have been mildly lengthened a few days before death, but again the changes have not been dramatic.

TABLE 20
SURVIVAL OF BEAGLES GIVEN CONTINUOUS
WHOLE-BODY γ IRRADIATION

R/Day	Approximate Total Dose, R	Dog No.	Survival from Be- ginning of Exposure,* Days
300.....	3300	686	13
210.....	2310	687	12
147.....	1617	688	15
103.....	1133	666	17
		689	22
72.....	792	667	21
		698	22
		699	24
		701	18
50.....	550	685	>24
		703	>24
		704	>24
		705	>24
Control....	None	669	
		683	
		684	

* The interval of observation, to date, is only 24 days.

Analysis of the changes leading to the lengthening of the prothrombin times are being attempted using the method of Mandel.⁽²⁾ Preliminary results suggest a deficiency of factor VII (SPCA).

Necropsy lesions in the decedents to this time have largely consisted of hemorrhages of varying degrees in a wide variety of tissues. Organ weights are being recorded, and tissues are being preserved and processed for microscopic examination.

REFERENCES

1. Quick, A. J. The prothrombin in hemophilia and obstructive jaundice. *J. Biol. Chem.* **109**, LXXIII (1935).
2. Mandel, E. E. 1962. Laboratory diagnosis of hemorrhagic disorders. *Clinical Diagnosis by Laboratory Methods*, ed. J. Davidson and B. B. Wells. Saunders, Philadelphia, 1962, pp. 322-370.

CERIUM-144—TOXIC MANIFESTATIONS IN BEAGLE DOGS

William P. Norris, Louise S. Lombard, Carl E. Rehfeld, and Norbert D. Kretz

PURPOSE AND METHODS

A total of 49 pure-bred beagles approximately 13 months of age was given Ce^{144} intravenously. The isotope was injected in sodium citrate at pH $\sim$ 4.0. Measurements of total retention were made at intervals using a NaI(Tl) crystal, γ -ray spectrometer. The doses of Ce^{144} ranged from 22.8 to 253.0 μ Ci/kg of body weight. This range of doses produced radiation effects from acute lethality down to delayed induction of tumors.

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TABLE 21—SURVIVAL OF BEAGLES FOLLOWING A SINGLE INTRAVENOUS DOSE OF Ce^{144}

Dog No.	SEX	DATE INJECTED	AGE AT INJECTION, DAYS	AMOUNT		DAYS TO DEATH	SURVIVAL AT 9-22-64, DAYS	CAUSE OF DEATH
				μ Ci	μ Ci/Kg			
132.....	M	8-10-60	680	2100	253.0	17		Bone marrow aplasia
119.....	M	8-10-60	680	2000	248.8	18		Bone marrow aplasia
112.....	M	8-10-60	772	1800	153.8	23		Bone marrow aplasia
129.....	M	8-10-60	649	1400	150.5	33		Bone marrow aplasia
261.....	F	4-18-62	500	956.0	122.7	0		Euthanized
1.....	M	12- 6-60	420	1086	120.0	26		Bone marrow aplasia
2.....	M	12- 6-60	420	995.0	102.0	31		Bone marrow aplasia
3.....	M	12- 6-60	420	833.0	96.4	53		Bone marrow aplasia
580.....	F	3-24-64	416	797.0	80.3		182	
548.....	M	1-15-64	410	823.4	79.2		251	
544.....	F	1-15-64	410	760.1	78.0	57		Bone marrow aplasia
547.....	M	1-15-64	410	624.6	77.9		251	
183.....	M	2-11-64	75	698.0	73.4	48		Hypoplasia of bone marrow
594.....	F	3-24-64	406	681.0	74.0		182	
585.....	M	3-24-64	416	979.0	72.8		182	
595.....	F	3-24-64	406	819.0	72.7	46		Bone marrow aplasia
589.....	M	3-24-64	416	761.0	72.5	89		Bone marrow aplasia
597.....	F	3-24-64	406	739.0	71.0		182	
598.....	M	3-24-64	406	953.0	69.0	56		Bone marrow aplasia
586.....	M	3-24-64	416	1098.0	67.1	60		Bone marrow aplasia
583.....	F	3-24-64	416	775.0	66.0		182	
565.....	M	2-11-64	415	705.0	64.0		224	
578.....	M	2-11-64	405	629.0	63.3		224	
516.....	M	11-27-63	412	606.0	62.9		300	
520.....	M	11-27-63	412	589.1	62.2		300	
521.....	F	11-27-63	412	571.6	62.1		300	
564.....	F	2-11-64	415	619.0	60.9		224	
567.....	F	2-11-64	408	739.0	59.9	99		Bone marrow aplasia
562.....	F	2-11-64	415	550.0	59.5		224	
519.....	M	11-27-63	412	626.9	58.9		300	
518.....	F	11-27-63	412	460.8	58.6		300	
517.....	M	11-27-63	412	481.9	55.2		300	
9.....	M	1-10-61	395	499.0	55.1	1158		Osteogenic sarcoma
7.....	M	1-10-61	395	463.0	53.0	1182		Reticulum cell sarcoma of vertebrae
11.....	M	1-10-61	395	439.0	49.7		1351	
12.....	M	1-10-61	395	410.0	49.4	995		Osteogenic sarcoma
13.....	M	1-10-61	395	394.0	48.3	1178		Osteogenic sarcoma
30.....	M	2-21-61	388	465.0	47.2	90		Bone marrow aplasia
22.....	M	2-21-61	410	436.0	47.1	77		Bone marrow aplasia
21.....	M	1-24-61	392	281.0	28.4		1337	
14.....	M	1-24-61	396	240.0	28.1		1337	
16.....	M	1-24-61	396	246.0	27.8	376		Hepatic necrosis and bone marrow aplasia
17.....	M	1-24-61	396	281.0	27.8		1337	
15.....	M	1-24-61	396	216.0	27.5		1337	
20.....	M	1-24-61	392	287.0	27.4		1337	
32.....	M	2-21-61	388	243.0	25.1		1319	
31.....	M	2-21-61	388	198.0	24.8		1319	
26.....	M	2-21-61	408	194.0	23.8		1319	
33.....	M	2-21-61	388	176.0	22.8		1319	

PROGRESS REPORT

The details of the doses of Ce^{144} given these dogs are presented in Table 21, where the animals are arranged in order of the dosages (in $\mu Ci/kg$ body weight) they received. The primary cause of death as determined at necropsy and by microscopic examination of tissues is also given for each animal which has died.

One will note that an intravenous dosage of $100 \mu Ci/kg$ is approximately the 30-day mid-lethal dose. At this dosage all animals died primarily of bone marrow aplasia. At lower dosages (down to $47 \mu Ci/kg$) bone marrow aplasia was the dominant cause of death. As the dosage was decreased from $100 \mu Ci/kg$ to $47 \mu Ci/kg$, the time of survival increased and presumably the development of marrow aplasia was correspondingly delayed. So far, however, no animals have died of marrow aplasia at times later than 100 days after Ce^{144} administration.

Animals living longer than 100 days have, with one exception, died of osteogenic sarcoma. This latter end point has been quite prevalent in the dogs given about $50 \mu Ci/kg$.

There has been little indication of hepatic injury, as judged by clinical examination, in any of these dogs. This is in spite of the fact that a substantial fraction of the injected Ce^{144} (40–60% of the injected dose) was found in the liver at the time of death.

The rate of uptake of Ce^{144} by the liver, as measured with a collimated NaI(Tl) crystal, was very rapid. The liver content of Ce^{144} stabilized at the end of 1–2 minutes after injection. The total-body retention of Ce^{144} was likewise very high, and physical decay was more important to its over-all loss than was physiological elimination.

RED CELL LIFE-SPAN IN THE BEAGLE STUDIED BY THE USE OF Fe^{59}

Lillian V. Kaspar, William P. Norris, and Norbert D. Kretz

PURPOSE AND METHODS

The life-span of the canine erythrocyte has not been investigated in much detail. This value is potentially useful in the evaluation of radiation effects on the hematopoietic system.

The method chosen is one which has been used successfully by Sacher and co-workers in mice. Briefly, a population of young erythrocytes was labeled by a single intravenous administration of ferrous citrate into a donor beagle. Seven days later these labeled red cells were withdrawn and transfused into three recipient beagle dogs. These animals were measured at intervals to determine their whole-body content of Fe^{59} .

PROGRESS REPORT

The life-span of the red cell, using this technique, is

determined by the time of inflection of the Fe^{59} retention curve. The iron tracer was retained quantitatively for approximately 80 days. After this time the data indicate a small but appreciable loss of Fe^{59} , and it appears that 80 days is a fair approximation of the life-span of the beagle erythrocyte. However, the data require a more detailed analysis.

The rate of Fe^{59} loss after 80 days was very low. Consequently, the expected inflection in the iron retention curve was not clearly evident until measurements of retention had been continued for a total of 120 days or more.

This technique requires too great an expenditure of effort to be useful in the evaluation of radiation effects in dogs. Alternatives are under consideration.

HEMATOLOGY OF THE BEAGLE AND EARLY EFFECTS OF CERIUM-144 RADIATION

Carl E. Rehfeld, Donald E. Doyle, William G. Richard,* James W. Bacus, and William P. Norris

PURPOSE AND METHODS

In the twelve-month period beginning in October 1963 four phases of the hematology of the beagle have made satisfactory progress. A scaler and a 400-channel pulse height analyzer were made to operate satisfac-

torily as units of a modified Coulter counter for routine blood cell enumeration and for erythrocyte sizing. The number of preexperimental hemograms has approached a number sufficient to provide hematologic standards which can serve as one basis for selection of beagles to be used in toxicology. Peripheral blood studies conducted with beagles given Ce^{144} , including employment

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of the 400-channel pulse height analyzer, have provided a basis for evaluation of the effect of Ce^{144} on erythropoiesis. A pilot study of the effects of continuous exposure of dogs to a Co^{60} source has provided some preliminary data regarding its effect on leukogenesis.

PROGRESS REPORT

Hematology Techniques

Conventional hemocytometry was employed initially and interpretation of the data indicated that erythropoiesis was sufficiently altered by Ce^{144} to result in a definite macrocytosis. A Coulter counter with a cell size analyzer was then employed to gain accuracy in cell enumeration; however, the cell size analysis was unsatisfactory. The third method which has been employed for the past year (since October 1963) retains the accuracy in cell enumeration and gives a much improved method for erythrocyte size analysis.

It was necessary to invest considerable time and effort in the development of a technique that allowed the modified Coulter counter to be used to produce routine hemograms plus size distribution curves. The Coulter principle involves drawing blood cells suspended in an electrolyte solution through a small aperture. Electrodes are placed on either side of the aperture, and electron flow is decreased momentarily by the presence of relatively nonconducting blood cells in the aperture. Decrease in electron or current flow is directly proportional to cell size which can be translated into proportional electrical pulses so pulse size is directly proportional to cell size. The 400-channel analyzer can be used in four segments of 100 channels or pulse recording compartments and four successive analyses stored for comparison and recovered as numerical values. Each pulse of several thousand representing as many cells is stored according to its relative size in one of the 100 channels in a one-minute sampling period.

The cell diluting fluid, aperture tube characteristics, aperture current settings, electronics, effect of current on the blood cells, the use of the oscilloscope, and use of the 400-channel pulse height analyzer were major considerations in establishing a routine procedure for the analysis of blood. The oscilloscope was particularly useful in monitoring the analyzer input pulses. The analyzer has been used in connection with Ce^{144} toxicity studies to demonstrate alterations in erythrocyte size and progressive variation in cell numbers per unit of blood. However, the scaler is used routinely for accurate cell enumeration and the analyzer only for size distribution studies. It is obvious, at this point, that this method of electronically counting and sizing peripheral blood cells is useful in toxicology and may permit the employment of smaller groups of dogs to obtain hematology data which can be used with confidence.

Hematology Standards

The establishment of hematology standards for use in connection with this beagle colony has now reached a point where these standards can be used as one vital determinant in selection of dogs for experimental work. The standards are made up of hemograms obtained at weekly intervals immediately prior to placement of the dogs in experimental groups at 13 months $\pm$ 2 weeks of age. Three successive hemograms are obtained, and the numerical results are averaged to represent a composite hemogram for each dog. Only those composite hemograms representing 3 or 4 preexperimental hemograms obtained between 12 and 14 months of age are added into the total number, which are again averaged to constitute standards for a certain period of time or for a certain hematology technique. In 1961, 1962, and early 1963 it was not always possible to arrange for three preexperimental hemograms from each dog so relatively small numbers of composite hemograms were included in those listings and averages. However, the three successive lists or sets of standards are now grouped according to the technique employed in obtaining the various blood values.

Conventional hemocytometry was used to obtain the composite blood values for 23 dogs from 10-5-61 through 5-14-62. A Coulter counter Model B was used for the second phase for a total of 34 dogs during the period 10-25-62 through 6-13-63. Using the same criteria for selection, a total of 89 dogs is included in the third group when the modified Coulter method was used from 10-21-63 through 8-31-64. The dogs are not separated on the basis of sex or genetic background, and the resultant average blood values which are differentiated on the basis of technique and time are listed in Table 22.

Hemocytometry and electronic cell enumeration were compared in a series of 231 blood samples from 12-10-63 through 3-17-64. The data from both methods were quite satisfactory, and either set could have been used; however, those from the electronic method indicated without doubt that this method was superior. The counts by the electronic method are highly reproducible, resulting in smoother curves and eliminating the necessity of trying to explain periodic abnormal rise and fall in cell numbers. Therefore, large numbers of samples are not necessary in order to counteract variations resulting from the counting technic, and a smaller number of dogs can be utilized with equal results.

There is no obvious explanation for the variation in results when comparing the three sets of control values. However, another comparison might be made if one divides the large third group into heterozygous and inbred dogs which number 74 and 15 for the total of 89 (Table 23). This comparison may not be appropriate

because of the difference in the sizes of the two groups, but a proportionately larger number of inbred dogs will be used in experiments with the passage of time.

Hematology in Cerium-144 Toxicity Studies

The effect of a moderately large dose (average 65.0 $\mu\text{Ci/kg}$) of Ce^{144} as seen in the peripheral blood has been studied for more than six months. A total of 24 dogs was used, including 6 which served as controls. Intravenous injection of the isotope was carried out at four different times as four groups of dogs reached the age of 13 months $\pm$ 2 weeks, but for purposes of studying the data the dogs were divided into two groups of 9 trial and 3 control dogs in each study group.

between 50 and 70 days after intravenous injection of the Ce^{144} . Recovery to preexperimental levels was obvious at around 115 days for both the hematocrit and hemoglobin content, but erythrocyte numbers had not recovered fully at 180 days. Recovery in terms of total circulating hemoglobin occurred by release of larger erythrocytes which contained the normal percentage of hemoglobin per unit weight of erythrocyte. Macrocytosis, the presence of larger than normal erythrocytes, was demonstrated also with the size distribution curves from the pulse height analyzer; however, the treatment of this information is only in a preliminary phase. Incidental to the use of the pulse height analyzer in following the course of changes in Ce^{144}

TABLE 22
HEMATOLOGY STANDARDS, SECOND GROUP OF DOGS

Series No.	No. of Dogs	Sampling Period	Av. Age, Days	Hematocrit, %	Hemoglobin, g/100 ml	RBC $\times 10^3/\text{mm}^3$	WBC/ mm^3
1.....	23	10-5-61-5-14-62	386	47.85	16.70	7,421	14,208
2.....	34	10-25-62-6-13-63	378	49.88	15.96	6,902	11,307
3.....	89	10-21-63-8-13-64	393	53.03	17.51	8,016	11,359
Total..	146		Av...389	51.48	17.02	7,663	11,796

TABLE 23
HEMATOLOGY STANDARDS, THIRD GROUP OF DOGS

No. of Dogs	Av. Age, Days	Hematocrit, %	Hemoglobin, g/100 ml	RBC $\times 10^3/\text{mm}^3$	WBC/ mm^3
74 (Heterozygous).....	393	53.23	17.70	8,071	11,631
15 (Inbred).....	398	52.04	16.57	7,745	10,017

The hematology to date has consisted primarily of a study of erythrocytes in the peripheral circulation plus a parallel enumeration of the leukocytes. Blood samples were obtained by venipuncture for determination of the hematocrit, hemoglobin (g/100 ml), erythrocyte numbers, and leukocyte numbers. The mean erythrocyte volume, mean erythrocyte hemoglobin, and mean erythrocyte hemoglobin concentration were then calculated according to the method of Wintrobe. Thrombocytes were enumerated by hemocytometry during the latter part of the study as time was available. In addition, four erythrocyte size distribution curves were obtained on each sample by means of a 400-channel pulse height analyzer.

The reaction of the first 9 experimental dogs to the Ce^{144} was quite consistent as interpreted from alterations in peripheral blood cells. Maximum depression of erythrocyte numbers and hemoglobin values occurred

toxicity has been the demonstration of microcytosis and macrocytosis in apparently normal nonexperimental dogs.

The hematology data from the second group of 9 experimental dogs have been quite different from those of the first group. A marked depression in circulating erythrocyte numbers occurred from 55 to 90 days with a correspondent decrease in hemoglobin, but hematocrit levels were not altered to the same degree. Inhibition of erythropoiesis was not as marked nor was macrocytosis apparent when values from the group were averaged. However, there was a significant increase in mean erythrocyte volume in 3 of these dogs, 2 of which died in the course of the experiment. Since 6 of the dogs in the first group had large numbers of macrocytes, 50% of the 18 dogs in the two experimental groups had circulating erythrocytes with a significant increase in mean cell volume.

ABSENCE OF THE TAPETUM LUCIDUM IN CERTAIN BEAGLES

Carl E. Rehfeld, James W. Bacus, and Calvin M. Poole

PURPOSE AND METHODS

The tapetum cellulosum is a membrane made up of several layers of cells which are roughly hexagonal in outline. This membrane is said to lie between the pigmented epithelium of the retina and the choroid or between the choriocapillary layer and vessel layer of the choroid depending upon the reference. When viewed with an ophthalmoscope the membrane is seen to be roughly triangular in shape with the apex dorsal and the base lying horizontally and at the level of the papilla. The appearance is striking because of the brilliant blue, yellow, and green colors reflected from it when light enters the fundus of the eye. Since light is reflected from the tapetum, visual acuity is improved particularly in comparative absence of light. The light sensitive retina benefits by the light entering the fundus and by that reflected from the tapetum which lies immediately posterior to the retina resulting in an intensified image. The tapetum is avascular, and the pigmented epithelium and perhaps the pigmented choriocapillary layer which lie between the light source and the tapetum are nonpigmented in the healthy young animal. In the absence of the tapetum, as in man, the light rays which pass through the retina are largely absorbed by the heavily pigmented choroid.

Certain dogs appear to lack the tapetum cellulosum. The tapetum may be thin and nonreflecting, or it may be similar to the tapetum fibrosum of ungulates, since the tapetum is not apparent. If the tapetum is lacking in these dogs their eyes should be very similar to the human eye anatomically except that there is a smaller amount of light-absorbant pigmentation in the fundus, or the vascular choroid is more apparent since light reflected from the fundus is reflected from a rich vascular bed. A logical deduction is that the atapetal eye is very similar to the human eye anatomically with the difference being lack of pigment in the pigment epithelium of

the retina and also in the choriocapillary layer of the choroid in that area overlying the usual position of the tapetum.

PROGRESS REPORT

Eight of the 9 atapetal beagles which have been identified in this colony were born in the kennels assigned to the study of the metabolism and toxicity of radionuclides. The 1 atapetal male was purchased years ago and was not used for breeding purposes until recent attempts were made to mate him with one of the atapetal females. This mating did not result in a pregnancy although the female has since produced two litters.

The 8 young atapetal beagles were born in six litters in which a total of 29 survived to an age when it could be determined whether or not the tapetal membrane was present. All 8 of these dogs were sired by only 2 males, these males being parent and offspring. All of the parents themselves possess the tapetal membrane.

The first 2 atapetal beagles noted were sired by the same male so he and one of his male offspring were used for subsequent matings pending appearance and survival of an atapetal male. Sufficient genealogical background information is not available to outline all parental relationships; however, it is doubtful whether any of the atapetal dogs mentioned here have an inbreeding coefficient greater than 0.0781.

Ra^{226} , Ra^{228} , and Th^{228} are all capable of destroying the cells which make up the tapetum lucidum. The effect of these radionuclides upon the fundus of the eye lacking the tapetum has not been determined by experimental means since beagles which lack the tapetum have not been readily available in laboratories studying radium toxicity. Radioactive isotopes of elements ordinarily found in the eye and which are of importance in its function could be studied with the aid of atapetal dogs.

THE EFFECTS OF FRACTIONATED DOSAGES OF RADIATION UPON SURVIVAL OF THE HIBERNATING GROUND SQUIRREL

Bernard N. Jaroslow and Douglas E. Smith

PURPOSE AND METHODS

Because of its markedly low body temperature and metabolism, the hibernating mammal is a useful tool for studying the development of radiation damage. Although the initial energy transfer reactions are not likely to be influenced by the range of temperatures involved in hibernation, one can expect that the subsequent chemical reactions would be temperature dependent. Thus, the development of damage leading to mortality is markedly slowed following lethal dosages of radiation to hibernating animals as compared with homeotherms.⁽¹⁾ In fact, when 80 to 100% lethal dosages are delivered during hibernation and the animals are kept in the cold for 3-4 weeks, no mortality occurs during this period. If the irradiated hibernators are then taken to and kept in a room at 23° C, they rouse from hibernation and mortality ensues on about the same schedule as if the animals had been irradiated on the day of their entry into the warm room. The purpose of the present experiments was to determine the possibility for recovery from a fractionated lethal dosage of radiation given during hibernation of the irradiated ground squirrel *Citellus tridecemlineatus*. The general methods employed in the irradiation and handling of these animals have been described in previous reports.⁽²⁾

PROGRESS REPORT

The following experiment was carried out by one of us (B. N. J.) in February and March of 1964 on animals that had been collected in the fall of 1963. Some of them were used previously in an immunological experiment, but had never been irradiated and were randomly distributed throughout the several groups of the present experiment. Three groups of ground squirrels were used in this study. Group A was exposed to 1200 R of X-rays while hibernating at 5° C, kept in the cold room for two weeks and then transferred to a room at 23° C where they remained thereafter. Group B was hibernating at 5° C and exposed to total-body X-ray

doses of 400 R one week apart. One week after the second dose they were removed to a 23° C environment where they were given a third dose of 400 R. They were kept at 23° C thereafter. Group C was always kept in the room at 23° C and given three dosages of 400 R at intervals of one week. The results of the experiments are shown in Table 24. No animals died during hibernation; all mortality took place upon removal of animals to the 23° C environment.

TABLE 24
EFFECTS OF FRACTIONATED DOSAGES OF RADIATION UPON SURVIVAL OF HIBERNATING GROUND SQUIRREL

GROUP	NUMBER OF ANIMALS	PER CENT SURVIVAL		
		30 Days*	60 Days*	180 Days*
A.....	14	27	27	20
B.....	16	94	87	87
C.....	16	62	56	44

* Days in 23° C environment after receiving total dosage of 1200 R.

CONCLUSIONS

It appears from these data that almost complete recovery occurs between split doses of radiation during hibernation (Group B). In fact, it seems that more recovery occurs from these fractionated doses than from similar doses to ground squirrels always kept at 23° C. There is no ready explanation for these results, and a repetition of this experiment will be attempted as part of a more elaborate study of fractionated radiation dosages on ground squirrels in the winter of 1964-65.

REFERENCES

1. Smith, D. E. The effects of ionizing radiation in hibernation. *Mammalian Hibernation*, ed. C. P. Lyman and A. R. Dawe. Bull. Museum Comparative Zool. 124, 493-506 (1960).
2. Jaroslow, B. N. and D. E. Smith. Antigen disappearance in hibernating ground squirrels. *Science* 134, 734-735 (1961).

DELAYED HYPERSENSITIVITY TO TRICHINELLA SPIRALIS ANTIGENS IN THE GUINEA PIG

Charles W. Kim

PURPOSE AND METHODS

Specific antibodies, elaborated by the host as a result of infection with *T. spiralis*, have been detected in circulating blood by various investigators (see review by Larsh⁽¹⁾). Immunity to *T. spiralis* has been shown to be acquired passively by the transfer of hyperimmune serum, and it has been transferred from mother to offspring (see review by Kagan⁽²⁾). However, there has been no demonstration of the true tuberculin type of delayed hypersensitivity to *T. spiralis*. Therefore, it seemed desirable to study the delayed hypersensitivity to the parasite in the guinea pig.

Male and female albino guinea pigs were sensitized with acid-soluble protein fraction and crude saline extract of the parasite.⁽²⁾ They were skin tested 7, 10, and 14 days following sensitization and later challenged with a shocking dose of the antigen to detect antibodies.⁽³⁻⁵⁾ Stretch preparations were made of the loose connective tissue from the injected sites at 2, 4, 6, 18, 24, and 28 hours following the skin test and stained with hematoxylin-eosin-azure II to study the cellular picture.⁽⁶⁾ Those guinea pigs giving a strong delayed skin reaction were used as donors in the splenic cell transfer to determine whether the hypersensitive state could be transferred adoptively to a normal recipient.^(7, 8)

PROGRESS REPORT

The results show that a typical delayed hypersensitive reaction to *T. spiralis* larval antigens (acid-soluble protein fraction and crude saline extract) can be developed in the guinea pig as shown by: 1) a delayed skin reaction of the tuberculin type in response to the antigen in combination with Freund's complete adjuvant that begins at 4 hours and reaches its peak in 18 to 24 hours; 2) the absence of circulating antibody when

animals are challenged intravenously with a shocking dose of the antigen at the peak of sensitivity; 3) a cellular infiltration into the skin test site that is primarily mononuclear in character (lymphocytes, monocytes, macrophages, and transitional forms); and 4) hypersensitivity that can be transferred to normal guinea pigs with splenic cells from sensitized donors, as manifested by a positive delayed skin reaction and characteristic cellular response.

CONCLUSIONS

The results have established the fact that a true delayed hypersensitivity to trichina antigens is acquired under the conditions of these experiments. Work is in progress to determine whether the mechanisms involved in delayed hypersensitivity are functional in acquired immunity and, if so, what their relation to antibody formation is.

REFERENCES

1. Larsh, J. E., Jr. *Advances in Parasitology*. Academic Press, Inc., New York, 1963.
2. Kagan, I. G. Trichinosis: A review of biologic, serologic and immunologic aspects. *J. Infectious Dis.* **107**, 65-93 (1960).
3. Gell, P. G. H. and B. Benacerraf. *Advances in Immunology*. Academic Press, Inc., New York, 1961.
4. Pappenheimer, A. M. and J. Freund. *Cellular and Humoral Aspects of the Hypersensitive States*. Paul B. Hoeber, Inc., New York, 1959.
5. Chase, M. W. *Cellular and Humoral Aspects of the Hypersensitive States*. Paul B. Hoeber, Inc., New York, 1959.
6. Taliaferro, W. H. and W. Bloom. Inflammatory reactions in the skin of normal and immune canaries and monkeys after the local injection of malarial blood. *J. Infectious Dis.* **77**, 109-138 (1945).
7. Chase, M. W. The cellular transfer of cutaneous hypersensitivity to tuberculin. *Proc. Soc. Exptl. Biol. Med.* **59**, 134-135 (1945).
8. Taliaferro, W. H. and L. G. Taliaferro. Immunologic unresponsiveness during the initial and anamnestic Forssman hemolysin response. *J. Infectious Dis.* **110**, 165-200 (1962).

DURATION OF DEOXYRIBONUCLEIC ACID SYNTHESIS IN MYELOID-ERYTHROID PRECURSORS

Peeyush K. Lala

PURPOSE AND METHODS

Replication of DNA is an essential prerequisite for mitosis in somatic cells. Since this process occupies a specific interval in the generation cycle of most cells; measurement of DNA-synthesis time (t_s) provides an

important tool in the study of cell cycle kinetics. This has been achieved so far in a variety of cell lines by a number of techniques.⁽¹⁻⁷⁾ The most recent one is a double labeling with H^3T and $C^{14}T$,^(6, 7) which depends on the differential in the energy range of the β particles

from H^3 and C^{14} as demonstrated on autoradiograms. This method for measuring t_s *in vivo* has thus far been applied as a single injection of H^3T followed shortly by a single injection of $C^{14}T$.⁽⁶⁾ In the present work, t_s *in vivo* for myeloid-erythroid precursors in marrow was measured in a more definitive way by an *in vivo* labeling with H^3T followed at short intervals thereafter by an *in vitro* labeling with $C^{14}T$. An estimation of t_s *in vitro* was also made by first labeling with H^3T *in vitro* following by relabeling with $C^{14}T$ at short intervals thereafter and vice versa. This technique may also be used to compare the cell fluxes into or out of DNA synthesis, on which the computation of t_s is based.

Table 25 shows the subjects and doses of labeled thymidine used in the different types of experiments.

To measure t_s *in vivo* (HC' type of experiment), H^3T was injected intravenously. About 0.7 ml bone marrow

vial and incubated for 30 minutes at 37° C. After centrifugation, cells were washed twice with culture medium and the volume brought to 4.5 ml with homologous plasma. Aliquots of 0.5 ml were now transferred into each of 4 pairs of culture tubes containing 0.5 ml culture medium and incubated at 37° C. At intervals of 1, 2, 3, and 4 hours each from the beginning of reincubation, $C^{14}T$ was added to the contents of one tube in a pair and the incubation allowed for another 30 minutes. Following this, both the cultures in a pair were taken out, and the cells were washed and resuspended for smear as earlier. In the C'H' type experiment the procedure was identical, except that $C^{14}T$ was substituted for H^3T and vice versa. Marrow from the same dog (A36G) was used for both types of *in vitro* experiments, aspirations being separated by an interval of one month.

TABLE 25
SUBJECTS AND DOSES OF LABELED THYMIDINE USED IN DIFFERENT EXPERIMENTS

SPECIES	SUBJECT	CONDITION	AGE IN YEARS	TYPE OF EXPERIMENT	DOSE OF H^3T IN μCi^*		DOSE OF $C^{14}T$ IN $\mu Ci^\dagger$ PER ML OF CULTURE <i>in Vitro</i>
					Per Kg Body Weight <i>in Vivo</i>	Per Ml Culture <i>in Vitro</i>	
Man	O. W.	N [†]	74	HC'	100		2.0
	A. K.	AML	85	HC'	100		2.0
Dog	B113	N	2.0	HC'	250		2.0
	A47A	N	3.0	HC'	250		2.0
	A36G	N	2.5	HC'	250		2.0
				H'C'		2.0	2.0
				C'H'		2.0	2.0

* Specific activity = 360 $\mu Ci/mmole$.

† Specific activity $\approx 25 \mu Ci/mmole$.

N, hematologically normal; AML, acute myeloid leukemia with myeloblasts forming 90% of peripheral leukocyte pool; HC', H^3T *in vivo* followed by $C^{14}T$ *in vitro*; H'C', H^3T *in vitro* followed by $C^{14}T$ *in vitro*; C'H', $C^{14}T$ *in vitro* followed by H^3T *in vitro*.

was then aspirated (from the sternum in man and proximal end of femur in dog) under local anesthesia into an equal volume of sterile homologous heparinized plasma at 30, 50, 70, and 90 minutes after H^3T injection. Immediately 0.5 ml of the sample was transferred into two culture tubes containing 0.5 ml of HUT-15 liquid culture medium* and $C^{14}T$; the rest of the marrow was used for immediate smears. Cultures were incubated at 37° C for 30 minutes, after which the cells were spun out, washed twice with Ringer's solution and then resuspended well in 4-5 drops of 22% bovine albumin for smears.

To measure t_s *in vitro* either H^3T was followed by $C^{14}T$ (H'C' type of experiment) or preceded by $C^{14}T$ (C'H' type of experiment). In H'C' type, about 2.5 ml marrow (pooled from 2 aspirations) aspirated into an equal volume of plasma was immediately transferred into 5 ml culture medium containing H^3T in a culture

Bone marrow and culture smears were prepared for autoradiographs with either NTB-3 or NTB nuclear emulsion (Eastman Kodak Co.). A single layer of undiluted emulsion warmed to 45° C was applied with no attempt to drain off the excess emulsion. Autoradiographs were exposed at 4° C for 4 weeks with NTB-3 and 8 weeks with NTB. At the appropriate time, autoradiographs were developed and stained with Giemsa.

One to three thousand cells including mitoses were scored at each point for each cell type in each case, except for myeloblasts in normal human or canine marrow, where the score ranged from 250-500 myeloblasts. A cell containing four or more grains was scored as positive when NTB-3 emulsion was used; with NTB emulsion, the threshold was taken as two grains. If the developed silver grains were on a single plane in close contact with the nuclei, the cells were considered to be labeled with H^3 alone. However, if the developed silver grains were present on more than one plane, these cells

* Kindly provided by the group of Dr. Warren K. Sinclair.

were scored as containing either C^{14} alone or $C^{14} + H^3$, henceforth referred to as C^{14} cells as no distinction could be made between the two groups. In the latter case, grains were also usually scattered in tracks beyond the boundary of the nuclei.

FIG. 79

FIG. 80

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In HC' type experiments, the labeling index with H^3T was determined for all the marrow samples before incubation with $C^{14}T$. In the samples after incubation with $C^{14}T$, labeling indices for different cell classes were determined for H^3 alone and for C^{14} or $C^{14} + H^3$. These data were used to calculate the per cent of the cohort in

FIG. 81

FIGS. 79-81.—Fluxes into or out of DNA synthesis *in vivo* shown for canine myelocytes, myeloblasts, and erythroid precursors.

synthesis (S) that has moved out of synthesis (S_0) or into (S_i) as follows:

$$S_0 = \frac{N_{H^3}}{N_{C^{14}}} \times 100,$$

where N_{H^3} = Number of cells labeled with H^3 alone after *in vitro* incubation with $C^{14}T$

and $N_{C^{14}}$ = Number of cells labeled with C^{14} or $C^{14} + H^3$ after *in vitro* incubation with $C^{14}T$;

$$S_i = \frac{LI(vitro) - LI(vivo)}{LI(vivo) \text{ at } 30 \text{ min}} \times 100,$$

where $LI(vitro)$ = Over-all labeling index after *in vitro* incubation with $C^{14}T$

and $LI(vivo)$ = Labeling index *in vivo*.

A plot of S_0 and S_i with time after H^3T injection provides the efflux from synthesis (K_0) or influx into synthesis (K_i) expressed as per cent per hour. If the system

remains in a steady state during the experimental period, K_o should equal K_i and $t_s = 100/K_o$ or $100/K_i$. As the fraction of telophases was small even at 90 minutes after H^3T injection, division of labeled cells would make negligible contribution to the above measurements.

FIG. 82

FIG. 83

FIGS. 82 AND 83.—Movement into or out of DNA synthesis *in vivo* shown for myelocytes, myeloblasts, and erythroid cells in hematologically normal marrow.

Although labeling *in vivo* and *in vitro* are combined, this method measures the flux of cells *in vivo*. Since the cells are labeled initially *in vivo* and the cells are sampled at various times thereafter, cells will have entered or finished synthesis *in vivo*. Those no longer in S phase will contain only H^3 label *in vitro*. Those entering synthesis will incorporate $C^{14}T$ *in vitro*, if viable, and cause an over-all increase in labeling index. That these cells remain viable is evidenced by an agreement between H^3T index *in vivo* and C^{14} index *in vitro*. Even if the rate of *in vitro* incorporation of precursor is different

FIG. 84.—*In vivo* progress into or out of DNA synthesis indicated for myeloblasts and erythroid precursors in the marrow of the patient with acute myeloid leukemia.

from the rate of *in vivo* incorporation, the same bias will be entered into each *in vitro* sample. Thus, since cells were sampled at four intervals, the rates of increase reflect *in vivo* synthesis of DNA and provide more precise information about t_s than with sampling at only one interval.^(5, 6) Figures 79–81 indicate S_o and S_i *in vivo* with time after H^3T administration for canine myelocytes, myeloblasts, and young erythroid cells, respectively. *In vivo* data for the hematologically normal individual are shown in Figures 82 and 83 and for the leukemic patient in Figure 84.

In the completely *in vitro* system, S_o or S_i *in vitro* is given by $N_{H^3}/N_{C^{14}} \times 100$, where N_{H^3} = Number of cells labeled with H^3 alone after incubation with the

second isotope; $N_{C^{14}}$ = Number of cells labeled with C^{14} or $C^{14} + H^3$ after incubation with the second isotope. S_0 is furnished by the $H'C'$ experiment and S_i by the $C'H'$ experiment as indicated in Figure 85.

No significant difference between the slopes of S_0 and S_i , i.e., between K_0 and K_i is noticeable in any of the above studies. DNA synthesis time (t_s) obtained from

FIG. 85.—Fluxes into DNA synthesis ($C'H'$ type experiment) or out of DNA synthesis ($H'C'$ type experiment) shown for canine myelocytes and erythroid cells *in vitro*.

$100/K_0$ or K_i is given for different cell types in the normal human and canine marrow and the marrow of one case of acute myeloid leukemia in Table 26.

The results are in excellent agreement with a previously reported value of 5 hours for canine myelocytes^(1, 4) and a value of 5.9 hours for canine erythroid precursors derived by different techniques. Data from one hematologically normal man suggest that DNA synthesis times for the normal human myelocyte, myeloblast, and erythroid precursor are not significantly different from the values in dog. When these t_s values and H^3T labeling indices are used to calculate the proliferation rates for myeloid and erythroid precursors in canine and human marrow, there is very close agreement with the rates derived from metaphase indices and a metaphase-time of 20 minutes in these cells.⁽⁸⁾

Measurements in one case of acute myeloid leukemia reveal no marked deviation in the synthesis times for

myeloblasts and erythroblasts in leukemic marrow from that in normal marrow. A low labeling index of myeloblasts with H^3T (6% in this patient) has been a consistent finding in acute myeloid leukemia.⁽⁹⁻¹¹⁾ Thus, this must reflect either a lengthening of other parts of the generation cycle or a large population of nonproliferating myeloblasts.⁽¹²⁾

There is a three-fold prolongation of synthesis times for both myelocytes and erythroid precursors in dog when measured *in vitro*. The agreement between the fluxes into and out of S measured in different ways indicates a true lengthening of the synthesis time *in vitro*, at least for the first generation, rather than a disturbance of cell movement into or out of S . Flash labeling indices at different times *in vitro* did not differ from the *in vivo* indices. This would mean a prolongation of the whole cell cycle *in vitro*. These findings are in good agreement

TABLE 26
DNA SYNTHESIS TIMES FOR MYELOID-ERYTHROID
PRECURSORS IN BONE MARROW
OF DIFFERENT SUBJECTS

CELL TYPE	SPECIES	SUBJECTS	DURATION OF S IN HOURS	
			<i>In Vivo</i>	<i>In Vitro</i>
Myelocyte	Man	O. W.*	6.1	
	Dog	B113	5.3	
		A47A	5.2	
		A36G	5.3	16.8
		Mean 5.3 ± 0.1		
Myeloblast	Man	O. W.*	5.7	
	Dog	B113	5.4	
		A47A	5.3	
		A36G	5.2	
		Mean 5.3 ± 0.1		
Erythroid pre- cursors	Man	O. W.*	6.0	
	Dog	B113	5.9	
		A47A	5.9	
		A36G	6.0	16.6
		Mean 5.9 ± 0.1		

* Hematologically normal individual.

† Acute myeloid leukemia.

with the times for marrow cells in cultures estimated by Lajtha some years ago⁽¹³⁾ with P^{32} and C^{14} labels in DNA.

CONCLUSIONS

A technique of double labeling with H^3 -thymidine *in vivo* followed by C^{14} -thymidine *in vitro* was developed to measure the *in vivo* period of DNA synthesis for myeloid erythroid precursors in the marrow of dogs, one hema-

tologically normal man and one case of acute myeloid leukemia. Results were in the approximate range of 5–6 hours. A completely *in vitro* measurement with H^3 -thymidine followed by C^{14} -thymidine and vice versa showed a three-fold prolongation of the synthesis time in canine myelocytes and erythroid precursors.

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REFERENCES

1. Patt, H. M. and M. A. Maloney. Kinetics of neutrophil balance. *The Kinetics of Cellular Proliferation*, ed. F. Stohlman, Jr. Grune and Stratton, New York, 1959, pp. 201–207.
2. Quastler, H. and F. G. Sherman. Cell population kinetics in the intestinal epithelium of the mouse. *Exptl. Cell Res.* 17, 420–438 (1959).
3. Leshner, S., R. J. M. Fry and H. I. Kohn. Age and the generation time of the mouse duodenal epithelial cell. *Exptl. Cell Res.* 24, 334–343 (1961).
4. Maloney, M. A., H. M. Patt and C. L. Weber. Estimation of

deoxyribonucleic acid synthetic period for myelocytes in dog marrow. *Nature* 193, 134–135 (1962).

5. Evensen, E. Significance of mitotic duration in evaluating kinetics of cellular proliferation. *Nature* 195, 718–719 (1962).
6. Pilgrim, Ch. and W. Maurer. Autoradiographische Bestimmung der DNS-Verdopplungszeit verschiedener Zellarten von Maus und Ratte bei doppel Markierung mit 3H - und ^{14}C -Thymidine. *Naturwissenschaften* 49, 544–545 (1962).
7. Wimber, D. E. and H. Quastler. A C^{14} - and H^3 -thymidine double labeling technique in the study of cell proliferation in *Tradescantia* root tips. *Exptl. Cell Res.* 30, 8–22 (1963).
8. Lala, P. K., M. A. Maloney and H. M. Patt. A comparison of two markers of cell proliferation in bone marrow. *Acta Haematol.* 31, 1–8 (1964).
9. Lajtha, L. G. On DNA labeling in the study of dynamics of bone marrow cell populations. *The Kinetics of Cellular Proliferation*, ed. F. Stohlman, Jr. Grune and Stratton, New York, 1959, pp. 173–182.
10. Unpublished data from this Laboratory.
11. Killmann, S. A., E. P. Cronkite, J. S. Robertson, T. M. Flidner and V. P. Bond. Estimation of phases of the life cycle of leukemic cells from labeling in human beings *in vivo* with tritiated thymidine. *Lab. Invest.* 12, 671–684 (1963).
12. Craddock, C. G. and G. S. Nakai. Leukemic cell proliferation as determined by *in vitro* DNA-synthesis. *J. Clin. Invest.* 41, 360–369 (1962).
13. Lajtha, L. G., R. Oliver and F. Ellis. Incorporation of ^{32}P and adenine- ^{14}C into DNA by human bone marrow cells *in vitro*. *Brit. J. Cancer* 8, 367–379 (1954).

GENERAL PURPOSE OF THE TISSUE MAST CELL PROGRAM

Douglas E. Smith

The mast cell characterized by Ehrlich in 1879 as a unique element of the connective tissue is round or elongate with a diameter of 20–30 μ and is packed with cytoplasmic granules ($\frac{1}{2}$ μ in diameter) which stain metachromatically with basic dyes such as toluidine blue. The cell contains and is the major source of heparin and histamine and, in some species, of 5-hydroxytryptamine. These compounds are of potential physiological significance in the normal animal and may cause various vascular-flow, clotting and permeability defects in the irradiated animal. Enzymes concerned with sulfation of heparin and decarboxylation of histidine, 5-hydroxytryptophane and dihydroxyphenylalanine are also found in the cell. Details of the work on the mast cell during

the last decade may be found in recent reviews by Mota⁽¹⁾ and Smith.⁽²⁾ Work in this laboratory has been centered on the morphology, origin, control and function of this little understood cell in the normal and irradiated animal. During the past year particular emphasis was given to the mast cell in antigen-antibody reactions, electron-microscopy of the developing mast cell and further experiments on the nature of its secretory activity. Reports of this work are presented in the full papers which follow.

REFERENCES

1. Mota, I. The behavior of mast cells in anaphylaxis. *Intern. Rev. Cytol.* 15, 363–397 (1963).
2. Smith, D. E. The tissue mast cell. *Intern. Rev. Cytol.* 14, 327–386 (1963).

THE ROLE OF THE MAST CELL IN ANAPHYLAXIS

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PURPOSE AND METHODS

Mast cells disrupt and release their cytoplasmic granules into the surrounding tissue when animals are exposed to a variety of treatments, e.g., adrenal cortical hormones, X-irradiation or antigen-antibody reactions.^(1, 2) Fatal anaphylaxis, difficult to produce in

rats, occurs in 90–100% of rats immunized with antigen plus *Bordetella pertussis* upon later challenge with specific antigen.⁽³⁾ Striking mast cell disruption and histamine release take place when tissues are immunized

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with horse serum plus *B. pertussis*, but not Freund's adjuvant, and later challenged with antigen.⁽⁴⁻⁶⁾

The purpose of the present experiments is to establish 1) whether mast cell disruption and histamine release follow passive antigen-antibody reactions using antisera from rats immunized with purified proteins plus *B. pertussis*, 2) the characteristics of the antibody responsible for mast cell disruption and histamine release, and 3) the role of this antibody in the fatal anaphylaxis of rats immunized with antigen plus *B. pertussis*. These studies were carried out on 200- to 350-g Wistar rats. Conventional immunological techniques were employed.⁽⁷⁻¹⁰⁾

PROGRESS REPORT

Mast cell disruption with release of histamine and 5-hydroxytryptamine may occur when samples of rat mesentery are incubated with rat antisera to various purified proteins (human serum albumin [HSA], bovine serum albumin [BSA], egg albumin [EA], etc.) and later exposed to antigen. It was established that the antibody bringing about this response is not the usual precipitating antibody and it has been called "mast cell sensitizing" antibody (MCSAb). Like human reagins, MCSAb is destroyed by heating at 56° C and when injected into homologous skin will remain at the site of injection for as long as one month. Its production is induced most effectively by an intraperitoneal or intradermal injection of *B. pertussis* organisms along with an intramuscular administration of the antigen. The best titers are obtained with EA. MCSAb seems to be a primary antibody, since its production is prevented or very much reduced by previous or additional contacts with antigens. The phenomenon of passive sensitization of mast cells with MCSAb was studied by incubating pieces of rat mesentery with antibody *in vitro*, using antigen-induced mast cell damage or histamine release as a measure of sensitization. Sensitization increases with time of contact between cells and antibody and can become maximum within a few minutes if the antibody concentration is high. Sensitization at 37° C and at 4° C proceeds at about the same speed. Nonspecific gamma globulin, when used in large amounts, may inhibit sensitization with MCSAb. Rabbit, rat, human, and bovine gamma globulin slowed down sensitization, whereas horse gamma globulin was without effect. Sensitization with MCSAb can be reversed completely by washing the sensitized mesentery with Tyrode's solution.

Passive cutaneous anaphylaxis (PCA) (as indicated by increased permeability to Evans blue dye) is induced with MCSAb, giving a maximal response 72 hours after administration of the antisera. This response may still be present 2 weeks after sensitization. The reaction seems to be due primarily to release of histamine and

5-hydroxytryptamine, since it is prevented by simultaneous administration of conventional antagonists to the action of these amines. Production of high titers of the usual precipitating antibody is brought about by repeated injections of the various antigens noted above. In these experiments 10 mg of HSA were given intramuscularly once a week for three months, followed by two injections of 3 mg of HSA a week apart. The sera contained 0.2 mg precipitating antibody per ml. They induced maximal PCA when challenged with antigen at six hours after administration. Mast cells were not disrupted in this reaction nor was the reaction prevented by histamine or 5-hydroxytryptamine antagonists. Severity of the PCA decreased with lapse of time between administration of precipitating antibody and challenge with antigen, the reaction being absent if challenge was delayed for 24 hours.

Rats inoculated with antigen and *B. pertussis* show severe shock which is 90-100% fatal when they are challenged about 10 days later with antigen. Treatment with antagonists of histamine or 5-hydroxytryptamine or prior depletion of the mast cell population does not protect the animals, strongly suggesting that MCSAb and damage to mast cells alone cannot account for anaphylactic death in this species. Rats with high titers of precipitating antibody do not show the severe and fatal anaphylaxis. In rats actively sensitized by the injection of antigen (EA) plus *B. pertussis* both immediate and delayed cutaneous anaphylactic reactions occur. Active cutaneous anaphylaxis (ACA) was induced in sensitized rats (known to contain MCSAb) by intravenous injection of Evans blue dye followed by small graded doses of specific antigen in the skin. Local dye leakage was measured upon killing the animals at $\frac{1}{2}$, 4, 24, or 48 hours later. Immediate ($\frac{1}{2}$ hour) ACA reactions occurred and could be inhibited by prior injection of histamine and 5-hydroxytryptamine antagonists. If animals pretreated with the antagonists were kept beyond 4 hours ACA did develop and was very severe by 24 and 48 hours. To assess the role of MCSAb and delayed hypersensitivity in systemic anaphylaxis the time course of their appearance was studied. It was found that severe systemic anaphylaxis and death could not be induced and MCSAb was not detectable in the sera of rats injected with *B. pertussis* and EA until after 6 days, whereas delayed ACA occurred upon injection of antigen as early as 4-5 days.

CONCLUSIONS

These experiments show that two types of antibody may be induced by the same antigen, depending on the regimen of immunization. Both can produce passive cutaneous anaphylaxis, one, MCSAb, by the apparent disruption of mast cells and release of histamine and

5-hydroxytryptamine; the other, the usual precipitating antibody, by mechanisms that are not clear. It appears that fatal anaphylaxis in rats occurs through additive or synergistic effects of immediate and delayed hypersensitivities.

REFERENCES

1. Mota, I. The behavior of mast cells in anaphylaxis. *Intern. Rev. Cytol.* 15, 363-397 (1963).
2. Smith, D. E. The tissue mast cell. *Intern. Rev. Cytol.* 14, 327-386 (1963).
3. Malkiel, S. and B. J. Hargis. Histamine sensitivity and anaphylaxis in the pertussis-vaccinated rat. *Proc. Soc. Exptl. Biol. Med.* 81, 689-691. (1952)
4. Mota, I. Mast cell lytic antibody. *Nature* 192, 1201 (1961).
5. Mota, I. Failure of rat and rabbit antiserum to passively sensitize normal and pertussis-treated rats and mice so as to induce mast cell damage and histamine release on later contact with antigen. *Immunology* 5, 11-19 (1962).
6. Mota, I. Mast cells and anaphylaxis. *Ann. N.Y. Acad. Sci.* 103, 264-277 (1963).
7. Mota, I. Biological characterization of "mast cell sensitizing" antibodies. *Life Sciences* 7, 465-474 (1963).
8. Mota, I. Passive cutaneous anaphylaxis induced with mast cell sensitizing antibody. The role of histamine and 5-hydroxytryptamine. *Life Sciences* 15, 917-927 (1963).
9. Mota, I. The mechanism of anaphylaxis. I. Production and biological properties of "mast cell sensitizing" antibody. *Immunology* 7, 681-699 (1964).
10. Mota, I. The mechanism of anaphylaxis. II. The role of "mast cell sensitizing" antibody and delayed hypersensitivity in rat anaphylaxis. *Immunology* 7, 700-706 (1964).

THE NATURE OF THE SECRETORY ACTIVITY OF THE MAST CELL

Douglas E. Smith, Marilyn A. Williams, and Yvette S. Lewis

PURPOSE AND METHODS

Mast cells appear to release their contents of heparin, histamine, and 5-hydroxytryptamine in several ways.⁽¹⁾ Treatment with X-radiation, cortisone, distilled water, and some histamine liberators bring about disruption of the cell and scattering of its cytoplasmic granules into the surrounding tissue. These changes are characteristic of holocrine secretion, and in their course the mast cells appear to be destroyed, for their number is markedly reduced and their restoration in the tissue takes many weeks.⁽²⁻⁴⁾ Apocrine secretion, characterized by loss of a portion of the mast cell granules, seems to occur in response to moderate dosages of one of the pharmacological histamine releasers⁽⁴⁾ and during the lipemic phase following a meal of fat.⁽⁵⁾ We have also suggested⁽¹⁾ that merocrine secretion occurs, since it appears that histamine and 5-hydroxytryptamine may be secreted without apparent damage to or release of cytoplasmic granules from mast cells subjected to low dosages of histamine liberators^(6, 7) or in the process of isolating free floating mast cells from the peritoneal cavity.^(8, 9)

Since there seems to be no general agreement on the nature of mast cell secretion,⁽¹⁾ it seemed worth while to subject the matter to a somewhat closer scrutiny than had been made heretofore. In this study the histamine content and the status of the mast cells in the windows of the mesentery of the rat were observed at various times following the intraperitoneal administration of toluidine blue or protamine sulfate. Both of these bind the heparin of the mast cell granule and promote the release of histamine. For histamine extraction the animals were sacrificed, and the mesenteries were removed, dissected and weighed in a room at 22° C and 73% relative humidity. Histamine was measured by bioassay using the ileum of the guinea pig. Samples of mesentery were

spread on slides and stained with toluidine blue for microscopic examination.

PROGRESS REPORT

Table 27 indicates that substantial reductions in histamine content are apparent in the mesentery of rats at 30 minutes after 20-ml dosages of toluidine blue (1:10,000 and 1:5,000 in Tyrode's solution). At 24 hours the histamine levels of the mesentery are partially and completely restored after 1:5,000 and 1:10,000 toluidine blue, respectively. Protamine sulfate seems to reduce the levels of histamine to a small extent at 30 minutes after a dose of 1:20,000 and to a great extent after a dose of

TABLE 27
HISTAMINE CONTENT OF MESENTERIC WINDOWS OF RAT FOLLOWING INTRAPERITONEAL ADMINISTRATION OF TOLUIDINE BLUE AND PROTAMINE SULFATE

TREATMENT	HISTAMINE EXPRESSED AS PER CENT OF CONTROL VALUES (IN $\mu\text{G/G}$ OF MESENTERY) FROM RATS SIMULTANEOUSLY ADMINISTERED TYRODE'S SOLUTION*	
	Time after Treatment	
	30 Minutes	24 Hours
Distilled HOH*	47†	18
Toluidine blue*		
1:20,000	97	92
1:10,000	76	100
1:5,000	23	56
Protamine sulfate*		
1:50,000	110	101
1:20,000	86	77
1:5,000	36	57

* All solutions administered in 20-ml amounts, the histamine releasers being dissolved in Tyrode's solution.

† Each figure is the average of analyses on at least six animals.

1:5,000. Recovery of histamine is not apparent at 24 hours after dosages of 1:20,000 but is suggested after the 1:5,000 dosage. There is considerable variation in histamine levels in these animals, and other experiments will have to settle the question of whether recovery actually occurs.

Mast cells in the mesenteric windows of untreated rats appear as round cells densely packed with large cytoplasmic granules. Occasionally in such animals one finds cells from which granules have been extruded into the surrounding tissue and phagocytes which have ingested mast cell granules nearby. A similar picture is found after the intraperitoneal injection of 20 ml of Tyrode's solution.

Treatment with distilled water brings about the complete disruption of mast cells, the cytoplasmic granules being widely dispersed into the surrounding tissue at 30 minutes. No phagocytosis of these granules is seen at this time. At 30 minutes after administration of 1:20,000 toluidine blue the mast cells look intact and normal, but significant numbers of phagocytes containing small metachromatic granules are found throughout many parts of the tissue. At 30 minutes following dosages of 1:10,000 and 1:5,000 toluidine blue mast cells are surrounded by few to many loose granules, respectively. Large numbers of phagocytes containing metachromatic granules are present in these mesenteric windows.

At 24 hours rats treated with Tyrode's solution show the picture described above for untreated animals. The mesenteric windows of rats treated with distilled water show practically no intact mast cells, a few granules loose in the connective tissue and large numbers of phagocytes containing metachromatic material. The tissues of animals given 1:20,000 toluidine blue appear normal; the mast cells are intact, and phagocytes containing metachromatic granules are rare. After 24 hours in animals given 1:10,000 toluidine blue the mast cells appear normal, but a few phagocytes with metachromatic granules are apparent. In the rats treated with 1:5,000 toluidine blue many mast cells look normal; some have loose metachromatic granules in their vicinity, and phagocytes containing metachromatic granules occur near both types of cell.

The histological picture following protamine administration is seldom uniform throughout any one of the several samples of mesentery from a single animal. Thus, at 30 minutes after dosages of 1:50,000 the mast cells generally appear normal except that in a few regions granules seem to have been released and phagocytes with granules appear nearby. After dosages of 1:20,000 the loss of granules is greater and phagocytosis of granules is also observed. Following 1:5,000 protamine loose granules were present in much greater numbers; phagocytes containing metachromatic granules appeared nearby. After all of these dosages the mast cells

appear normal except for the loss of granules. A striking feature of the above regions, however, is the occurrence of halos of metachromatic material about the mast cells; this occurs with greater frequency with the higher dosages. After one day such metachromatic halos are no longer seen, and the mast cells in all groups appear normal. Phagocytes with metachromatic granules still appear in some regions after the dosages of 1:5,000 and 1:20,000.

CONCLUSIONS

The results of the toluidine blue experiments strongly indicate that measurable reductions in the histamine content of the windows of the mesentery of the rat do not occur without the release of cytoplasmic granules from the mast cell. The appearance of significant numbers of phagocytes containing metachromatic granules within five minutes as well as at 30 minutes following 1:20,000 toluidine blue suggests that secretory products may be released from mast cells after this dosage also. The amounts of histamine that may be released may be too small to be detected by the methods employed. The morphological and biochemical recovery of the mast cells in the above experiments appears to reside in the individual mast cells rather than be the result of new mast cells. Mast cell numbers do not appear to increase, nor is division of mast cells observed.

The results of the experiments with protamine sulfate are not readily explained. This substance easily replaces histamine that is bound to heparin.⁽¹⁾ It readily enters the mast cell where its effects have been observed *in vivo*.⁽⁷⁾ After the stronger concentrations reductions in tissue histamine were apparent, and these were accompanied by the apparent loss of metachromatic material, which appeared in the form of a halo about the cell. The fate of this material is not clear for it is gone at the end of 24 hours and is not apparent in phagocytes.

REFERENCES

1. Smith, D. E. The Tissue Mast Cell. Intern. Rev. Cytol. **14**, 327-386 (1963).
2. Smith, D. E. and Y. S. Lewis. Effects of total-body X-irradiation on the tissue mast cell. Proc. Soc. Exptl. Biol. Med. **82**, 208-212 (1953).
3. Fawcett, D. W. Cytological and pharmacological observations on the release of histamine by mast cells. J. Exptl. Med. **100**, 217-224 (1954).
4. Fawcett, D. W. An experimental study of mast cell degeneration and regeneration. Anat. Rec. **121**, 29-52 (1955).
5. Hill, M. Secretion of heparin by mast cells. Nature **180**, 654-655 (1957).
6. Smith, D. E. Nature of the secretory activity of the mast cell. Am. J. Physiol. **193**, 573-575 (1958).
7. Smith, D. E., Y. S. Lewis, and G. Svihla. Cinephotomicrography of living mast cells under the influence of pharmacological agents. Anat. Rec. **136**, 359 (1960).
8. Uvnäs, B. and I. L. Thon. Isolation of biologically intact mast cells. Exptl. Cell Research **18**, 512-520 (1959).
9. Garcia-Arocha, H. Release of histamine and 5-hydroxytryptamine from cells of the peritoneal fluid of rats. Canad. J. Biochem. Physiol. **39**, 395-402 (1961).

THE ULTRASTRUCTURE OF THE TISSUE MAST CELL

Douglas E. Smith, Sally F. Vierling, and Yvette S. Lewis

PURPOSE AND METHODS

A knowledge of the morphological details of the tissue mast cell is a necessary basis for the understanding of its functional dynamics, especially the production and release of its physiologically active secretory products. Convincing evidence concerning the existence of ordinary cellular constituents such as mitochondria, Golgi apparatus, and limiting membranes has not emerged from more than eighty years of light microscopy of the mast cell.

In recent years the use of the electron microscope in our hands⁽¹⁻³⁾ and in those of others (for review see Reference 4) has done much to delineate the fine structure of the mast cell under normal and experimental conditions, including X-irradiation.⁽¹⁾ Thus, we have

established that these cells contain mitochondria,⁽¹⁾ limiting membranes,⁽¹⁾ endoplasmic reticulum,^(1, 2) Golgi apparatus,⁽³⁾ centrioles,⁽³⁾ and different types of cytoplasmic granules.⁽³⁾ The purpose of our study is the further delineation of the ultrastructure of the mast cell under normal and experimental conditions including exposure to various types of radiations. During the past year particular attention has been paid to the development of the mast cell utilizing tissues from early foetal to two-month-old Sprague-Dawley rats. Tissues have been fixed in osmium tetroxide or a mixture of osmium tetroxide and potassium permanganate,⁽⁵⁾ embedded in Epon 812 resin, sectioned with the Huxley microtome and stained with uranyl acetate and lead citrate alone or in sequence prior to examination in the Siemens Elmiskop I electron microscope.

FIG. 86.—Mast cell in skin of paw of 16-day foetal rat. In all figures arrows indicate cytoplasmic granules

FIG. 87.—Mast cell in skin of paw of 19-day foetal rat

FIG. 88.—Mast cell in skin of nose of 8-week-old rat

PROGRESS REPORT

A clear-cut sequence of developing mast cells has been found in sections of the free-floating peritoneal cells and in sections of the skin of the paw and of the nose (Figures 86-88). Thus, in early foetal rats mast cells with but a few cytoplasmic granules are seen. The remainder of the cytoplasm shows signs of an actively synthesizing cell, i.e., well-developed Golgi apparatus, endoplasmic reticulum, nuclear pores, mitochondria, ribosomes, etc. The cytoplasmic granules are found within spaces in the endoplasmic reticulum. In older foetal and in newborn rats gradations of mast cells are seen in the same preparations. Thus, cells with a single row of granules peripherally and with a rich cytoplasm about the nucleus occur along with cells with more granules and less visible cytoplasm. In older rats the mast cell with little or no apparent cytoplasm and a multitude of closely packed cytoplasmic granules is almost completely dominant. Autoradiography is being used in combination with electron microscopy to establish the locus of and steps in the formation of the heparin-protein backbone of the cytoplasmic granules as well as of the formation of histamine, 5-hydroxytryptamine, and several other substances reputed to be made in the mast cell.

In collaboration with M. Kollmorgen we have examined recently ultrathin sections of the P815Y subline (grown in tissue culture and in the peritoneal cavity of the mouse) of the original Dunn-Potter mouse mastocytoma. The ultrastructure of the cells in tissue cultures under conditions of optimal growth resembles that for the Furth,⁽⁶⁻⁹⁾ Rask-Nielsen and Christensen^(10, 11) and

the closely related Dunn-Potter mouse mastocytoma.^(7, 8) A striking difference is the almost complete lack of typical cytoplasmic granules in the P815Y cells in tissue culture.

REFERENCES

1. Smith, D. E. and Y. S. Lewis. Electron microscopy of the tissue mast cell. *J. Biophys. Biochem. Cytol.* **3**, 9-14 (1957).
2. Bloom, G., B. Larsson and D. E. Smith. The reaction of peritoneal mast cells in the rat to various activators. *Acta Pathol. Microbiol. Scand.* **40**, 309-314 (1957).
3. Smith, D. E. Electron microscopy of normal tissue mast cells under various experimental conditions. *Ann. N.Y. Acad. Sci.* **103**, 40-52 (1963).
4. Smith, D. E. The tissue mast cell. *Intern. Rev. Cytol.* **14**, 328-386 (1963).
5. Tahmisian, T. N. Use of the freezing point method to adjust the tonicity of fixing solutions. *J. Ultrastruct. Res.* **10**, 182-188 (1964).
6. Hagen, P., R. V. Barnett and F. L. Lee. Biochemical and electron microscopic study of particles isolated from mastocytoma cells. *J. Pharmacol. Exptl. Therap.* **126**, 91-108 (1959).
7. Bloom, G. Studies on the cytology, cytochemistry and pathology of the mast cell. *Diss. med.*, Stockholm, (1960).
8. Bloom, G. Electron microscopy of neoplastic mast cells: A study of the mouse mastocytoma mast cell. *Ann. N.Y. Acad. Sci.* **103**, 53-86 (1963).
9. Sellyei, M. and H. David. Untersuchungen über die Ultrastruktur einer Variante des Furthsehen Mäusemastozytoms. *Zeitschr. Mikroskop. Anat. Forschung.* **70**, 103-117 (1963).
10. Christensen, H. E., O. H. Iversen and R. Rask-Nielsen. Studies on a transplantable mastocytoma in mice. II. Electron microscopic observations. *J. Natl. Cancer Inst.* **30**, 763-781 (1963).
11. Iversen, O. H., H. E. Christensen and R. Rask-Nielsen. Studies on a transplantable mastocytoma in mice. III. Histologic and ultrastructural following varying doses of oestrogen. *Acta. pathol. microbiol. Scand.* **60**, 194-208 (1964).

IN VITRO MARROW PERFUSION

Robert L. Straube

PURPOSE AND METHODS

In an attempt to maintain bone marrow cells in an isolated, yet functionally active preparation, we have devised an *in vitro* perfusion system for the long bones of small mammals.

The donor animal is heparinized, anesthetized with sodium pentothal, and exsanguinated by cardiac puncture. The two femurs are disarticulated, muscles removed, and the foramen on the medial surface of the shaft just below the lesser trochanter, through which the main nutrient artery and vein enter, is enlarged with a bone drill in one femur. A rubber dam, drawn taut with wound clips, is placed over the foramen to prevent seepage, and pierced by an 18-gauge, non-beveled needle. The femur is enclosed within a modified

50-ml glass syringe and a siliconized soft rubber tube passes from the drip reservoir through a sigmoid pump to the needle in the nutrient foramen. About 7-8 ml of heparinized plasma fills the system, which is suspended in a warm box (37° C). When the pump is activated, plasma enters the marrow cavity via the foramen, traverses the marrow and escapes through the cortex via the innumerable small foramina. A flow of 2-2.5 ml per minute is maintained. Samples (2-3 ml) are withdrawn from the drip reservoir at hourly intervals and replaced with an equal amount of cell-free plasma. From the samples thus removed, cell number is determined on the Coulter electronic cell counter, and deoxynucleoprotein is precipitated with hot 5% TCA. Aliquots are taken for DNA spectrophotometric determination by

the diphenylamine reaction and radioactivity determination of H^3 content by scintillation counting (Tricarb). Total femur cell content is obtained by splitting the cortical bone, carefully removing all marrow, making a homogenous suspension in physiological saline and counting in the Coulter electronic cell counter.

The total marrow cell content of the right and left femurs of the Dutch belted female rabbit is the same within 3-4%. Occasionally, gross foci of necrosis are encountered. Such marrows are discarded. The marrow cell content is a function of both body weight and age, but the internal control (opposite femur) obviates the necessity of such corrections in individual experiments.

Fifty per cent Micropaque or Ilypaque was injected intravenously in anaesthetized rats using a polyethylene catheter inserted high in the femoral artery. X-ray films were taken within one minute after the completion of the injection. These films were compared to those of femurs mounted on the perfusion apparatus and perfused with an identical opaque mixture. In both cases the material could be seen within the marrow cavity in discreet threads of varying thickness passing through the marrow, suggesting that it lay within preexisting vascular sinusoids, rather than disrupting the structure of the marrow and filling the cavity indiscriminately.

After 4-5 hours of perfusion, from 10 to 90×10^6 myeloid cells are released from the marrow to the perfusate. Since the total cell content of perfused and control marrows are within normal limits, it is presumed that this cell excess has been produced by continuing proliferation within the marrow.

PROGRESS REPORT

The wide variation in cell yield may reflect the influence of cell concentration on cell release from the perfused marrow. There is some indication in these preliminary experiments that the concentration of cells in the perfusate must periodically be dropped below a given value before maximum subsequent release of cells is obtained. Continuous infusion with acellular plasma might be preferable to the closed system employed, but large volumes of plasma would be needed.

MODIFICATIONS OF ANTIBODY FORMATION

William H. Taliaferro, Lucy Graves Taliaferro, and Bernard N. Jaroslow

PURPOSE AND METHODS

For some years, we have been trying to quantitate the effect of X-radiation on antibody formation in rabbits by varying the time of irradiation, the dose of radiation and of antigen, etc.⁽¹⁻³⁾ In addition, the extreme radiosensitivity of the nucleic acid phases of antibody forma-

tion occurring during the early part of the process has made possible the separation of induction per se from the elaboration of the antibody-synthesizing mechanism.⁽⁴⁻⁶⁾ Therefore, we have extended our radiation studies to include the ability of oligonucleotides to restore induction and antibody formation when the latter

When H^3T is injected 2 hours before sacrifice, the specific activity of the marrow greatly exceeds that of the cells in the perfusate; with injection 1 or 2 days before sacrifice, the specific marrow activity decidedly lags behind the activity of the cells in the perfusate. These findings suggest that the preparation approaches the physiological norm in that mature cells are released to the perfusate from the marrow before immature dividing cells. Sacrifice within a few hours of H^3T injection would find the label in a cohort of immature dividing cells normally unlikely to be released until the unlabeled marrow reserves were exhausted; with 1-2 days' interval between labeling and sacrifice the cohort of labeled cells would have advanced into the mature marrow reserves and thus be more readily released to the perfusate.

Such preliminary findings indicate the general feasibility of the technique, but a number of problems remain. Work is in progress on extending the life of the preparation and clarifying the basic relationships of cell release.

CONCLUSIONS

An apparatus for the *in vitro* perfusion of marrow contained in the isolated long bones of small mammals is described. The pattern of distribution of radiopaque material within the femoral marrow space is similar, as seen on X-ray film when a) the material is injected *in vivo* via the femoral artery or b) by the perfusion apparatus. In the rabbit, the right and left femurs have the same marrow cell content within 4%. When one femur is perfused and the other serves as a control, the cell content of the two femurs is within the same limits after 4-5 hours of perfusion. In addition, about 30×10^6 (10 - 90×10^6) cells accumulate in the perfusate. If the donor rabbit is injected with H^3T (1 mCi/kg) 24 or 48 hours before sacrifice, the specific activity of the cells in the perfusate exceeds that of the marrow; if the donor rabbit is injected two hours before sacrifice, the specific activity of the marrow greatly exceeds that of the perfusate.

tion occurring during the early part of the process has made possible the separation of induction per se from the elaboration of the antibody-synthesizing mechanism.⁽⁴⁻⁶⁾ Therefore, we have extended our radiation studies to include the ability of oligonucleotides to restore induction and antibody formation when the latter

are intensely damaged by X-rays and have begun studies on the effect of nucleic acid analogues on antibody formation.

The model used has involved the hemolysin response in rabbits injected with sheep red blood cells because it can be accurately measured by various parameters, such as peak titer and the rate of synthesis. A 50% hemolysin end point is determined by a photoelectric colorimeter, as described by us previously.^(7, 8)

PROGRESS REPORT

Effect of Radiation on Hemolysin Production

The purpose of this work was to examine the effect of the time of radiation 1) with respect to the time of injecting antigen and 2) with respect to varying the X-ray dose. A study was made of 72 groups of rabbits injected initially with $10^{8.3}$ sheep red cells per kg rabbit at eight intervals from 2 days before through 2 months after one total body irradiation of nine different doses of X-rays from 25 to 700 R.

The data show that the time of injecting this amount of antigen with respect to a given dose of X-ray may profoundly enhance or depress the hemolysin response. On the one hand, enhancement of the response, as gauged by high peak titers, is accompanied by a true stimulation (a rapid rate of rise to peak titer) when antigen is injected 2 days or 2 hours before or 1 month after small doses of X-rays (25 to 100 R) and is accompanied by a retardation of the response when antigen is injected at the same intervals before or after large doses of X-rays (500 to 700 R). On the other hand, marked depression of the response, as gauged by low peak titers and low rates, occurs when antigen is injected one or two days after the large doses of X-rays. This work has been published.⁽⁹⁾

The Restoration of Hemolysin Production in X-rayed Rabbits

We have found that antibody induction and hemolysin-forming capacity lost in previously X-rayed rabbits can be restored to varying degrees by injecting rabbits with partially depolymerized heterologous nucleic acids (oligonucleotides) at the time that the red cells were injected.⁽⁴⁻⁶⁾ Since polymerized nucleic acids from heterologous sources were ineffective, we have collected homologous DNA from spleens of normal rabbits and from rabbits sensitized by sheep red cell injections.

We are studying the restorative action of these materials on hemolysin formation. This work is in progress, and the results have not been formulated as yet.

The Effect of Nucleic Acid Analogues on the Hemolysin Response

It has been reported⁽¹⁰⁾ that 5-bromodeoxyuridine (5 Br) and other base analogues may be incorporated into DNA of various cells in place of the natural component thymidine and that such cells become highly susceptible to various types of irradiation.

The action of 5 Br has been studied in our model with and without irradiation. Large doses have been injected intraperitoneally into rabbits at the rate of 0.1 to 0.25 g at intervals of 45 minutes before to 2 hours after the injection of red cells into normal rabbits and also into rabbits irradiated with 400 and 500 R the day before or the day after.

Although several large groups of rabbits have been studied, the results are inconclusive, apparently because the compound greatly increases the variability of the hemolysin response.

REFERENCES

1. Taliaferro, W. H., L. G. Taliaferro and E. F. Janssen. The localization of X-ray injury to the initial phases of antibody response. *J. Infectious Dis.* **91**, 105-124 (1952).
2. Taliaferro, W. H. and L. G. Taliaferro. Effect of X-rays on hemolysin formation following various immunization and irradiation procedures. *J. Infectious Dis.* **95**, 117-133 (1954).
3. Taliaferro, W. H. and L. G. Taliaferro. Further studies on the radiosensitive stages in hemolysin formation. *J. Infectious Dis.* **95**, 134-141 (1954).
4. Jaroslow, B. N. and W. H. Taliaferro. The restoration of hemolysin-forming capacity in X-irradiated rabbits by tissue and yeast preparations. *J. Infectious Dis.* **98**, 75-81 (1956).
5. Jaroslow, B. N. Factors associated with initiation of the immune response. *J. Infectious Dis.* **107**, 56-64 (1959).
6. Taliaferro, W. H. and B. N. Jaroslow. The restoration of hemolysin formation in X-rayed rabbits by nucleic acid derivatives and antagonists of nucleic acid synthesis. *J. Infectious Dis.* **107**, 341-350 (1960).
7. Taliaferro, W. H. and L. G. Taliaferro. The dynamics of hemolysin formation in intact and splenectomized rabbits. *J. Infectious Dis.* **87**, 37-62 (1950).
8. Taliaferro, W. H. and L. G. Taliaferro. X-ray effects on hemolysin formation in rabbits with spleen shielded or irradiated. *J. Infectious Dis.* **99**, 109-128 (1956).
9. Taliaferro, W. H. and L. G. Taliaferro. The relation of radiation dosage to enhancement, depression, and recovery of the initial Forssman hemolysin response in rabbits. *J. Infectious Dis.* **114**, 285-303 (1964).
10. Djordjevic, B. and W. Szybalski. Genetics of human cell lines. III. Incorporation of 5-bromo- and 5-iododeoxyuridine into the deoxyribonucleic acid of human cells and its effect on radiation sensitivity. *J. Exptl. Med.* **112**, 509-531 (1960).

ANTIBODY FORMATION

William H. Taliaferro and Lucy Graves Taliaferro

PURPOSE AND METHODS

Antibody formation is a particularly useful type of protein synthesis because of the specific reaction of antibody with the antigen that induces its formation. Earlier work by us involving isotopic labels⁽¹⁾ has helped to establish 1) that antibody formation is chemically a direct *de novo* synthesis from the amino acids of the free amino acid pool without the formation of any long-lived amino acid-containing precursors and 2) that the transit time is only about 30 minutes for isotopically labeled amino acids injected into the blood stream to be incorporated into antibody in significant amounts. These findings indicate that the changes in amount of antibody in the blood serum satisfactorily approximate the actual rate of synthesis *minus* the rate of decay of antibody.

We have also employed electrophoretic and immunological methods to characterize the antibodies that are formed in rabbits after immunization with sheep red blood cells.⁽²⁻⁴⁾ Of these, the two Forssman hemolysins have closely related, if not identical, specificity. They are a γ_1 macroglobulin antibody that is formed very quickly after immunization and a γ_2 antibody that is formed later. They differ in metabolic half-life, molecular weight, avidity of antigen-antibody union, and rate of hemolytic activity.

In our current work, we have used as a model of antibody formation the hemolysin response in rabbits because hemolysin can be assayed by the amount of hemoglobin it liberates from red cells and can be accurately measured with a photoelectric colorimeter. A 50% end point is used as described by us.^(5, 6) Important parameters of the response include peak titer and the rate of synthesis.

PROGRESS REPORT

Effect of Antigen Dosage on the Hemolysin Response

The primary object of this work has been to examine the effect of a wide range of antigen doses on the initial and anamnestic hemolysin responses. This study forms an important base line upon which experimental modifications of the two responses can be evaluated.

Typical sigmoid regression curves were constructed relating parameters of the hemolysin response to log antigen dose over a 4-million antigen range (10^4 to $10^{10.6}$ cells) for the initial response and over a 2.5-billion antigen range ($10^{1.2}$ to $10^{10.6}$ cells) for the secondary response.

The data indicate that as the antigen dose is increased, an antibody-synthesizing mechanism of increasing capacity develops in a shorter time and proceeds to synthesize more antibody in a shorter time. The most

likely assumption to explain these findings is that an increase in antigenic stimulation shortens the generation time—and possibly the induction and maturation times—of antibody-forming cells during the early part of the hemolysin response. This work has been published.⁽⁷⁾

Immunologic Unresponsiveness (i.e., Acquired Tolerance) to Forssman Antigen

Other authors (see reviews by Hasék et al.⁽⁸⁾ and Smith⁽⁹⁾) have reported immunologic unresponsiveness following the injection of large amounts of antigen other than the Forssman antigen. This unresponsiveness was acquired in marked antigen excess.

As these results are of importance in lowering antibody production, we were interested in repeating the experiment within the framework of our model. Because $10^{10.6}$ sheep red cells is about as large a dose of red cells as can be injected per kg rabbit we used intravenous and intraperitoneal injections of heated minced horse kidney, which also contains the Forssman antigen. Groups of adult rabbits, either irradiated previously with 500 R or not irradiated, received six injections a week for three weeks. Injections were then discontinued as some of the rabbits died at the beginning of the fourth week.

No immunologic unresponsiveness occurred in the irradiated or unirradiated groups, but the serums are being studied further.

REFERENCES

1. Taliaferro, W. H. General introduction: Synthesis and degradation of antibody. *J. Cellular Comp. Physiol.* **50** (Suppl. 1), 1-26 (1957).
2. Taliaferro, W. H., L. G. Taliaferro and A. K. Pizzi. Avidity and intercellular transfer of hemolysin. *J. Infectious Dis.* **105**, 197-221 (1959).
3. Taliaferro, W. H. and L. G. Taliaferro. Intercellular transfer of gamma-1 and gamma-2 Forssman hemolysins. *Proc. Natl. Acad. Sci.* **47**, 713-724 (1961).
4. Stelos, P., L. G. Taliaferro and P. A. D'Alesandro. Comparative study of rabbit hemolysins to various antigens. III. Chromatographic analysis of Forssman hemolysins induced by various antigens. *J. Infectious Dis.* **108**, 113-119 (1961).
5. Taliaferro, W. H. and L. G. Taliaferro. The dynamics of hemolysin formation in intact and splenectomized rabbits. *J. Infectious Dis.* **87**, 37-62 (1950).
6. Taliaferro, W. H. and L. G. Taliaferro. X-ray effects on hemolysin formation in rabbits with spleen shielded or irradiated. *J. Infectious Dis.* **99**, 109-128 (1956).
7. Taliaferro, W. H. and L. G. Taliaferro. The effect of antigen dosage on the Forssman hemolysin response in rabbits. *J. Infectious Dis.* **113**, 155-169 (1963).
8. Hasék, M., A. Lengerová and M. Vojtíšková (eds.). *Mechanisms of Immunological Tolerance*. Academic Press, Inc., New York, 1963.
9. Smith, R. T. Immunological tolerance of nonliving antigens. *Advances in Immunology* **1**, 67-130 (1961).

Animal Facilities Operations

DISEASE CONTROL PROGRAM

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

PURPOSE AND METHODS

The purpose of this program is to control the diseases of all the breeding, stock and experimental animals. The several rodent breeding colonies are continually monitored for the presence of microbial, helminthic, and metazoan pathogens. Rats and mice purchased from outside vendors are similarly screened for common bacterial pathogens and parasites. In addition, spontaneous decedents are necropsied to determine the cause of death. Many other control measures are also employed such as the maintenance of a continuous log of the cage and bottle washer operating temperatures, pressure and causticity; the culturing of sample clean cages and bottles; the operation of a continuous and effective vermin control program; and the maintenance of accurate records of disease incidence.

PROGRESS REPORT

During the period covered by this report, the monitoring of the breeding colonies was extended to include, in addition to tests for *Pseudomonas aeruginosa*, *Salmonella* sp., and internal and external parasites, tests for *Citrobacter freundii*, *Mycoplasma* sp. and *Pasteurella pneumotropica*. The program of necropsying spontaneous decedents was extended to include, in addition to the breeding colony, all stock animals and, whenever

possible, the animals in experiments as well. However, because of manpower limitations and the difficulty in interpreting the results of necropsies of animals in experiments, a great effort was made to differentiate carefully between experimental animals and stock animals. For this reason, all the animals in the facilities were rearranged in such a way that those not yet actually in experiments are housed together in stock animal rooms by species, strain, source and disease status rather than randomly distributed (by investigator) throughout the facilities as in the past. As an extension of this and as a part of the disease control program, the animals actually in experiments were also rearranged. Instead of being grouped together by investigator, the animals in experiments are now also grouped by species, strain, source and disease status. In combination with the relocation of the animals, various new identification, inventory, necropsy and data storage forms were developed. All were designed for use with the animal vital statistics computer program. This aspect of the program is not yet completed, but already it has been found that the information being acquired is of great value in controlling animal disease in the facilities. It will ultimately allow for better interpretation of experimental results.

SURVEY OF HEALTH STATUS OF COMMERCIALLY PRODUCED MICE

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

PURPOSE AND METHODS

The purpose of this study is to survey the health status of mice raised by commercial breeders.

Ten discarded breeding females and ten discarded breeding males of the Lynch/Webster or ICR strains are being purchased from eleven commercial breed-

TABLE 28—RESULTS OF SURVEY OF HEALTH STATUS OF MICE RAISED BY COMMERCIAL BREEDERS

BREEDER	NUMBER OF MICE EXAMINED	NASAL SWAB					FECAL CULTURE				ECTOPARASITES	ENDOPARASITES
		<i>Pasteurella pneumotropica</i>	<i>Pseudomonas aeruginosa</i>	<i>Proteus</i> species	PPLO*	Other	<i>Salmonella</i> species	<i>Pseudomonas aeruginosa</i>	<i>Citrobacter freundii</i>	Other		
A.....	10	9	3	2	0	0	0	3	0	0	0/10†	0/10†
B.....	10	0	0	3	0	1	0	3	0	0	0/11	0/10
C.....	10	4	0	10	0	0	0	3	0	0	1/12	6/10
D.....	10	5	0	0	0	0	0	4	0	0	15/15	3/10
E.....	10	6	1	8	1	0	0	1	0	3‡	10/10	5/10
F.....	10	10	1	0	4	2	0	0	0	0	9/10	5/10
Totals...	60	34	5	23	5	3	0	14	0	3	35/68	19/60

* Pleuropneumonia-like organisms.

† Number positive/number examined.

‡ Bethesda-ballerup organisms.

ers located throughout the country. Five animals of each sex are examined shortly after arrival at the Laboratory for ectoparasites, and five of each sex are necropsied and examined microbiologically for various potential pathogens and for endoparasites.

PROGRESS REPORT

Although the survey is only about half completed,

the results (Table 28) indicate that the incidence of some bacterial pathogens is low but that the incidence of others is high. The incidence of internal and external parasites is also high for most breeders. No attempt was made to survey for viruses at this time.

CITROBACTER FREUNDII ASSOCIATED WITH DIARRHEA IN LABORATORY MICE

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

PURPOSE AND METHODS

An outbreak of diarrhea among several hundred stock and experimental mice occurred during early 1964. The first sign of the disease was smearing of the cages of affected mice with loose feces. Affected animals were emaciated, lethargic, and showed pasting of the perineum and tail with feces. The mortality in affected groups was about 25–50%; the morbidity ranged from 80–90%. The gross lesions at necropsy were those of a catarrhal enterocolitis with an accumulation of mucus in the colon. The colon was dilated and the wall thickened and congested. No formed feces were found in any of the affected animals.

From all affected animals a nonmotile gram negative rod identified as an atypical *Citrobacter freundii* was isolated. It fermented glucose, lactose, and mannitol but not sucrose. It did not produce indole, hydrogen sulfide, hydrolyze urea or utilize citrate as a sole source of carbon. Since these reactions were significantly different from most strains of *C. freundii*, we have for purposes of convenience, called the Argonne strain *Citrobacter freundii*-Anl. This organism comprised 90–100% of the aerobic microflora isolated from feces, intestines, heart blood, and liver of affected animals.

To determine whether *C. freundii*-Anl was the cause of this disease, groups of 10 female CF#1/Anl mice four weeks of age and weighing 20–24 g were inoculated with a culture of *C. freundii*-Anl isolated from a mouse with diarrhea. A bacteria-free filtrate of homogenized gut and gut content from mice with diarrhea was also given.

Some groups were inoculated with the pure culture of *C. freundii*-Anl alone, some were given the filtrate alone, and some a combination of the two.

PROGRESS REPORT

Those that received pure cultures of *C. freundii*-Anl either alone or in combination with the filtrate developed clinical signs and lesions indistinguishable from the naturally occurring disease. The filtrate alone was without effect. Approximately 80% of the animals receiving *C. freundii*-Anl became ill, and about 40% died. All experimentally infected animals, both those that died and those that survived, yielded *C. freundii*-Anl on culture.

The disease, with a mortality comparable to that observed in natural outbreaks, was also produced by placing healthy mice free of the organism in contact with clinically affected animals. *C. freundii*-Anl was recovered from the newly affected animals.

CONCLUSIONS

It is concluded that *C. freundii*-Anl is the primary cause of the disease because of the fulfillment of Koch's postulates. It is further concluded that a virus is not the primary etiologic agent because none of the animals which received the filtrate alone developed the disease. The possibility that latent infection with one or more agents may be necessary for the expression of pathogenicity of *C. freundii*-Anl is not excluded by these results.

STUDIES ON THE PATHOGENICITY OF CITROBACTER FREUNDII-ANL

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

PURPOSE AND METHODS

In the recent epizootic of enterocolitis that occurred in our laboratory mice it was shown that *Citrobacter freundii*-Anl was the causative organism. Hitherto, organisms of this genus and species have been of question-

able importance as a cause of enterocolitis, and the question arose whether some change in the intraluminal environment had altered the pathogenicity. Furthermore it was found that in the majority of cases in the laboratory mice there had been an increase in the numbers of

Clostridium perfringens. We decided to test the following hypothesis. Infection with *C. freundii*-Anl resulted in a change in the intestinal lumen that allowed an increased growth of *C. perfringens*. Because of the ability of *C. perfringens* to split conjugated bile acids, the amount of bile acid metabolites increased. The raised level of free bile acids, such as deoxycholic and lithocholic acid, caused an increase in the secretion of mucin, and perhaps the diarrhea and damage to the mucosa. These cytotoxic acids may also cause the release of endotoxins or reduce the efficiency of the defense mechanisms. Three experiments were performed.

In Experiment 1, three groups of 10 CF#1/Anl male mice 40 days old were injected intraperitoneally with a) 0.3 ml of an 8-hr culture of *C. freundii*-Anl in trypticase

TABLE 29

EFFECT OF *C. freundii*-ANL AND DEOXYCHOLIC ACID GIVEN BY INTRAPERITONEAL INJECTION

	No. of Mice	No. of Mice Dead on Day 3	No. of Mice with Diarrhea
<i>C. freundii</i> -Anl.....	10	0	0
<i>C. freundii</i> -Anl and deoxycholic acid.....	10	10	10
Deoxycholic acid.....	10	0	3
Untreated controls.....	10	0	0

soy broth (about 10^7 organisms), b) the same as a) plus 0.5 ml of 0.25% deoxycholic acid given immediately after the injection of the bacteria and c) 0.5 ml of 0.25% deoxycholic acid.

In Experiment 2, 59 male CF#1/Anl mice maintained on a diet containing 1% sodium deoxycholate were given a suspension of *C. freundii*-Anl (about 10^8 viable organisms) a) intravenously, b) intraperitoneally, c) by stomach tube. This dose of *C. freundii*-Anl had previously been shown to be insufficient to produce the characteristic syndrome within 14 days. Sixty CF#1/Anl mice maintained on a standard diet as a control group were treated similarly to the experimental group. Cultures of heart blood were made from all animals either three or eight days after the introduction of the bacteria.

In Experiment 3, three groups of 10 germfree HA/ICR mice were given 0.2 ml of suspension containing 10^{11} viable organisms of *C. freundii*-Anl a) intravenously, b) intraperitoneally, c) by stomach tube. Groups of conventionally raised mice were similarly treated.

PROGRESS REPORT

The results of the experiments are shown in Tables 29-31. The combination of *C. freundii*-Anl and deoxycholic acid proved singularly lethal. No deaths occurred in any of the other groups. It was of interest that deoxycholic acid alone caused diarrhea in three of ten mice. In Experiment 2 a large percentage of the mice maintained on the diet containing sodium deoxycholate

TABLE 30

NUMBER OF POSITIVE BLOOD CULTURES IN MICE ON A DIET CONTAINING SODIUM DEOXYCHOLATE OR A STANDARD DIET FOLLOWING INFECTION WITH *C. freundii*-ANL*

ROUTE OF INTRODUCTION	DIET WITH SODIUM DEOXYCHOLATE			STANDARD DIET		
	No. of Mice	3 Days	8 Days	No. of Mice	3 Days	8 Days
Stomach tube.....	20	9	4	20	0	0
Intravenous.....	19	9	6	20	0	0
Intraperitoneal.....	20	0	1	20	0	0

* Results given for three and eight days after infection.

TABLE 31

NUMBER OF MICE WITH DIARRHEA FOLLOWING INFECTION WITH *C. freundii*-ANL

ROUTE OF INTRODUCTION	GERMFREE		CONVENTIONAL	
	No. of Mice	No. of Mice with Diarrhea	No. of Mice	No. of Mice with Diarrhea
Stomach tube...	10	0	9	8
Intravenous...	10	0	10	6
Intraperitoneal..	10	0	10	1

which were given the organism either intravenously or by stomach tube showed positive blood cultures. In Experiment 3, no clinical signs or deaths occurred in the germfree mice infected with *C. freundii*-Anl as a mono-contaminant, whereas 15 of the 30 conventional mice showed signs and lesions.

These results suggest that the presence of at least one other organism is necessary for the expression of the pathogenicity of *C. freundii*-Anl. Further investigations will test what combinations of bacteria are pathogenic. It will then be possible to investigate the nature of the interaction. At this stage it is not possible to say the hypothesis is correct, but none of the results obtained so far contradict it.

MURINE PNEUMONIA PROBLEM

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

PURPOSE AND METHODS

Our previous work with *Pasteurella pneumotropica* and our continuing problems with chronic pneumonia in mice and rats have prompted us to investigate further the causes and predisposing factors of this disease.

We are isolating and collecting the microorganisms recovered from spontaneous cases of pneumonia as well as obtaining murine strains of pleuropneumonia-like organisms (*Mycoplasma* sp.) from several outside sources. These organisms are being tested alone or in combination with other organisms and predisposing or stress factors to determine their pathogenicity.

PROGRESS REPORT

In many spontaneous cases of murine pneumonia, *P. pneumotropica* can be isolated in pure culture with no evidence of other microorganisms, but to date we have been unable to reproduce pneumonia in mice using this organism. The inoculation of mice with *Mycoplasma* sp. or filtrates of pneumonic lung tissues alone has also been generally unsuccessful. We now plan to try to reproduce the disease by inoculating various combinations of these three possible agents.

HEMORRHAGIC DIATHESIS IN LABORATORY RODENTS

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

PURPOSE AND METHODS

The spontaneous occurrence of a hemorrhagic diathesis in newly established colonies of specific-pathogen-free rats and mice within the last year has led us to investigate the cause and predisposing factors as well as methods for preventing the problem. Although hemorrhagic problems have been reported previously in laboratory rodents, most of these have been associated with irradiated foods or experimental diets. One reported spontaneous outbreak in mice was identical to the syndrome we have observed in our animals. In this case ethylene oxide sterilization of bedding resulting in formation of toxic glycols was suggested as the cause of prothrombin-complex deficiencies resulting in spontaneous hemorrhage. The similarity of our problem to that associated with glycols by other investigators and our need for more knowledge regarding specific-pathogen-free animals and their maintenance and diseases has made it imperative that we conduct experiments to study blood clotting diseases and mechanisms in the laboratory mouse and rat.

PROGRESS REPORT

Approximately 10 weeks after the first deaths in our newly initiated CF#1/Anl-SPF and SD/Anl-SPF colonies, we treated both groups by giving weekly subcutaneous injections of 5 μ g of menadione sodium bisulfite (vitamin K) to $\frac{1}{3}$ of the animals in each colony. After 10 weeks of treatment, the ration being fed these animals was altered, and menadione was supplemented

in it at a rate of 2.83 ppm. The treatment with menadione was considered beneficial because five times as many animals died with hemorrhages in the untreated as in the treated groups.

Whole blood clotting times and one-stage prothrombin times were determined on a series of apparently normal conventional SD rats as well as on clinically affected and unaffected animals. The whole blood clotting times and one-stage prothrombin times of affected and unaffected SD/Anl-SPF rats were greatly increased compared to the conventional SD rats, but these abnormal findings were largely reduced to the normal range 24 hours after treatment with 5 μ g of menadione.

Differential diagnosis of the abnormal one-stage prothrombin times was also performed on the rat and mouse plasmas. This procedure indicated factor II (prothrombin) and VII (SPCA) to be deficient and factor X (Stuart) to be suspect. Factor V (AC-globulin) and fibrinogen were normal.

Microscopic examination of tissues from affected mice and rats revealed consistent myocardial lesions in all of the male mice affected with the syndrome but only an occasional lesion in the male rats and female mice and rats. The significance of menadione has become more apparent with this observation since Dessau et al.⁽¹⁾ have reported cardiac lesions in mice on vitamin K deficient diets.

Another interesting aspect of these studies has been the finding that female mice and rats are more commonly affected with the hemorrhagic syndrome during

pregnancy. In our studies, approximately $\frac{2}{3}$ of the females dying were pregnant, and the principal site of hemorrhage was the uterus and fetal membranes. Since it has been established by several investigators that the vitamin K requirements of the male rat are considerably higher than those of the female and that these requirements are sex hormone dependent; we suspect that the requirements of the female are increased by the hormonal changes of pregnancy.

Studies of the intestinal flora of animals dying of the hemorrhage syndrome have been inconclusive since all animals were carrying organisms known to be capable of synthesizing vitamin K. Because most rodents are thought normally to synthesize enough vitamin K to maintain themselves if they practice coprophagy to absorb intestinally produced vitamins, we are unable to explain the sudden appearance of the condition only in

our SPF breeding colonies. Although the problem has disappeared in the animals now supplemented with menadione in their food, it continues in that portion of the colonies still untreated with the severity varying from time to time. The reason for the fluctuation has not been but needs to be determined.

Studies will be continued on the relationship of vitamin K to the syndrome and the pathogenesis of the myocardial lesions. Other facets of this problem to be investigated are the predisposing factors which initiate the syndrome and attempts to reproduce it using better defined diets.

REFERENCE

1. Dessau, F. S., T. Lipchuck and S. Klein. Heart lesions in mice given diets deficient in vitamins E and K. Proc. Soc. Exptl. Biol. Med. 87, 522-524 (1954).

RODENT BREEDING PROGRAM

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

PURPOSE AND METHODS

This program was initiated to provide better, more highly controlled animals than are available from commercial breeders. At the present time, the program includes colonies of CF#1, CP (catalase positive), CN (catalase negative), C57L, A/JAX, LAF₁, HR (hairless) and Mastomys mice and SD rats.

A planned random-bred, monogamous mating system is used. All animals are weighed before issue and those not within the prescribed range are discarded. The special disease control procedures for the breeding colonies include the periodic testing of all breeding pairs for *Pseudomonas aeruginosa*, *Salmonella* sp., and *Citrobacter freundii*. Those found infected are discarded. In addition, all sick, moribund or dead animals and all young animals that are underweight are necropsied and the cause of illness or death determined if possible. Discarded breeders from the various colonies are also examined. These are necropsied, cultured for *Pseudomonas aeruginosa*, *Salmonella* sp., *Mycoplasma* sp. (PPLO) and *Pasteurella pneumotropica* and examined for internal and external parasites.

PROGRESS REPORT

During the period covered by this report, 6002 breeding room water bottles were cultured for the presence of *Pseudomonas aeruginosa*; 55 were positive. Thirty-five of the positive cultures were from the specific-pathogen-free rat colony initiated from stocks supplied by a commercial breeder. Of 1400 fecal samples examined for

Salmonella species, none was positive. Similarly, no positive cultures for *Citrobacter freundii* were disclosed. Results of screening of Argonne raised discarded breeding animals are shown in Table 32.

TABLE 32
SCREENING RESULTS OF ARGONNE RAISED
DISCARDED BREEDING RODENTS

Examination	No. positive/ No. examined	% Positive
<i>Pseudomonas aeruginosa</i> . . .	0/270	0
<i>Salmonella</i> species	0/270	0
<i>C. freundii</i>	0/270	0
PPLO (<i>Mycoplasma</i> sp.) . . .	0/270	0
Ectoparasites	8/270	2.9
Endoparasites	5/270	1.8

Of 1392 water bottles from purchased mice examined for *Pseudomonas* species, 264 were positive. Four of 88 purchased animals were positive for *Salmonella* species, and 65 of 151 purchased animals have been shown to harbor *C. freundii*.

The CF#1/Anl colony which produces the bulk of the animals used by the Division, by all tests, was completely free of *P. aeruginosa*, *Salmonella* sp., and *C. freundii*.

CONCLUSIONS

The mice raised by the Division are far superior to those available from commercial suppliers. The CF#1/Anl colony is the only colony known in the world to be completely free of *P. aeruginosa*.

DOG CARE PROGRAM

Calvin M. Poole and Robert J. Flynn

PURPOSE AND METHODS

The purpose of this program is to provide professional veterinary care for all the Division's dogs. The program provides for daily observations of all dogs; canine distemper-hepatitis vaccinations at 12 weeks of age, 4 months of age, 1 year of age, and annually thereafter; routine physical examinations every 4 months; routine dental examinations at 1 year of age and every 4 months thereafter; parasitological examinations at 6 weeks of age, 10 weeks of age, and every 4 months thereafter; examination, diagnosis, and treatment of all observed diseases or abnormalities; professional surgical assistance and postoperative care; and routine care of newborn pups.

PROGRESS REPORT

The general health status of the dog colony continues to be extremely good. There have been no contagious

diseases nor external parasites observed during the period covered by this report. The incidence of internal parasitism has also been low; only coccidia and one species of roundworm, *Toxascaris leonina*, have been observed.

The only major change in care procedures made during the period covered by this report is the initiation of an *ad libitum* feeding system to replace the previous method of giving a measured amount of feed for a limited time to each individual animal. The effects of the new method of feeding have been evaluated. Its advantages are 1) it requires less time and effort to feed, 2) it has significantly reduced the incidence of fighting, 3) it has reduced individual food competition, 4) it allows greater expression of the individual's phenotype, 5) it has resulted in a more uniform nutritional state in the colony. The disadvantages of the system are 1) it is more difficult to determine the status of an individual's

TABLE 33

COMPARATIVE WEIGHT GAINS OF 100- TO 200-DAY-OLD DOGS FED *ad Libitum* OR CONVENTIONALLY DURING THREE DIFFERENT 5-MONTH PERIODS

Dog Group	No. in Group	Average Age, Days	Average Beginning Wt., g	Average End Wt., g	Average Gain, g	Average Loss, g
Conv. system Feb. 1963 to July 1963...	10	140	7090	9808	2718	
Conv. system Sept. 1963 to Feb. 1964...	45	146	7705	10075	2370	
<i>Ad lib.</i> Feb. 1964 to July 1964...	33	138	6419	10759	4340	

TABLE 34

COMPARATIVE WEIGHT GAINS OF 200- TO 365-DAY-OLD DOGS FED *ad Libitum* OR CONVENTIONALLY DURING THREE DIFFERENT 5-MONTH PERIODS

Dog Group	No. in Group	Average Age, Days	Average Beginning Wt., g	Average End Wt., g	Average Gain, g	Average Loss, g
Conv. system Feb. 1963 to July 1963...	22	255	9345	10057	712	
Conv. system Sept. 1963 to Feb. 1964...	26	246	9991	9996	5	
<i>Ad lib.</i> Feb. 1964 to July 1964...	54	274	9518	11005	1487	

TABLE 35

COMPARATIVE WEIGHT GAINS OF 1- TO 2-YEAR-OLD DOGS FED *ad Libitum* OR CONVENTIONALLY DURING THREE DIFFERENT 5-MONTH PERIODS

Dog Group	No. in Group	Average Age, Days	Average Beginning Wt., g	Average End Wt., g	Average Gain, g	Average Loss, g
Conv. system Feb. 1963 to July 1963...	26	583	10261	11021	760	
Conv. system Sept. 1963 to Feb. 1964...	29	505	10229	10054		175
<i>Ad lib.</i> Feb. 1964 to July 1964...	30	530	9994	11235	1241	

TABLE 36

COMPARATIVE WEIGHT GAINS OF 2- TO 3-YEAR-OLD DOGS FED *ad Libitum* OR CONVENTIONALLY DURING THREE DIFFERENT 5-MONTH PERIODS

Dog Group	No. in Group	Average Age, Days	Average Beginning Wt., g	Average End Wt., g	Average Gain, g	Average Loss, g
Conv. system Feb. 1963 to July 1963...	25	902	9550	10337	487	
Conv. system Sept. 1963 to Feb. 1964...	37	955	10986	10263		723
<i>Ad lib.</i> Feb. 1964 to July 1964...	20	943	10289	11497	1208	

appetite, 2) it requires more advanced planning in order to restrict an animal's diet temporarily, 3) it has resulted in a general tendency toward obesity.

In order to evaluate the effects of *ad libitum* feeding more objectively, weights were tabulated for comparable groups of dogs for three different five-month periods (Tables 33-36). During two of these periods, February 1963 to July 1963 and September 1963 to February 1964, the dogs were on the conventional feeding system and for one period, February 1964 to July 1964, they were on the *ad libitum* system. The *ad libitum* system produced weight gains that were almost double those of

the conventional system. In addition, the weight gains for conventionally fed animals during the February 1963 to July 1963 period were much larger than during the September 1963 to February 1964 period. This difference cannot be readily explained except on the basis of seasonal variation. Further observations are being made.

Progress reports for two other aspects of the dog care program, the dog breeding program and studies of canine dental problems, are included with the report of the group concerned with the toxicity and metabolism of radionuclides.

ANIMAL VITAL STATISTICS COMPUTER PROGRAM

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

PURPOSE AND METHODS

The animal vital statistics program utilizing computer systems for data recording and processing is directed toward providing a method of more efficiently keeping rodent breeding records, providing a critical analysis of the breeding efficiency of the colonies, collecting data on the incidence of diseases, and providing a continuous check on the health status of animals intended for experimental use. It should also be helpful in noting trends and suggesting leads in the investigation of the more subtle or insidious causes of death or sickness in laboratory animals.

PROGRESS REPORT

All animals in the Division's rodent breeding facilities are now incorporated into this system, and data now being recorded include daily observations on all changes in the health status, births, deaths, weanings,

weaning weights, and culling. These data are analyzed weekly, and a summary is prepared that lists litters born, number of animals born, average litter size, number of young animals dead, sick, culled, or missing, number of litters weaned, number of animals weaned, average litter size weaned, total breeding pairs in production, and number of adult animals sick, dead, culled, or missing. Also included is a weekly listing of all young animals dying in the breeding colonies correlated with the date of death, the age in days at the time of death, and the cause of death. The program is now being expanded to include mortality data on all stock animals (animals issued from the breeding colonies but not yet actually in experiments). Also being developed for integration into this program is a necropsy record-keeping system which will consist of a multipart form designed for punch card recording and sorting of the data collected from necropsy examinations and clinical laboratory procedures including hematology and microbiology.

GROWTH AND DEVELOPMENT IN COMPENSATED FIELDS: INTRODUCTION AND DESIGN*

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Joseph Gvildys,† Robert Kleb,‡ George Halper,‡ William Cole,‡
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PURPOSE AND METHODS

Growth and growth phenomena, such as tropisms, are defined both by the structure of the organism and its constituents and by gradients in electrical charge across that structure. Both structure and gradient are, in turn, a consequence of polar differences induced sequentially at cell, tissue, and organ levels. There is evidence that such polarity is modifiable in plants by reorientation in gravitational, electrical, and magnetic fields. Hence, these oriented fields may be assumed to have a determinative effect on either the initiation or nature of the polarization. If the above assumption is corrected, it may be inferred that development in an abnormal or subthreshold field would result in alterations ranging from changes in anatomical structure to the extreme of acellular accretion.

This project was designed to determine if any changes occur at morphological, physiological, and biochemical levels of organization in plants subjected to continuous reorientation of field direction. By this approach we hope to derive some insight into the function of earth fields in plant morphogenesis. We hope also to obtain bases for educated guesses about the behavior and development of terrestrial plants in extraterrestrial environments, with particular emphasis on radiation response.

The experimental plan required apparatus that would move differentiated and undifferentiated tissues about single and about two- and three-coordinate orbits simultaneously. The vector motion of the test object

would be such that the effect of a directional field would be continuously compensated for by shifts in orientation of that field in less than minimal presentation time. The present report summarizes the accomplishments and anticipations with three types of apparatus: one-coordinate (1π), two-coordinate (2π), and three-coordinate (4π) compensators.

PROGRESS REPORT

2π

Preliminary biological experiments have been run with the 2π apparatus illustrated in Figure 89. This apparatus provided compensation for earth gravitational and magnetic fields, at the same time imposing a centrifugal force on the test organism of about $5 \times 10^{-6} g$ in a plane normal to the vertical. *Arabidopsis* plants grown from seed in the apparatus grew more slowly than their uncompensated counterparts; at any given time, leaves were fewer and smaller, and floral initiation and development were delayed. On the other hand, vegetative replication of fronds in compensated *Spirodela* was ca. 25% greater than in the uncompensated controls. Elongation was significantly greater in the *Avena* coleoptile, mesocotyl, and root under compensation when the embryo of the seed was initially oriented toward the direction of increasing centrifugal force. With all three organisms, directions of organ growth appeared independent of spatial coordinates.

Light microscopy revealed no significant changes in gross cell morphology induced by 2π compensation of the above three organisms. It should be stressed, however, that in all instances the growth and development observed in these plants involved replication of pre-existing histological templates.

The results with the organs of the *Avena* seedling are

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† Applied Mathematics Division.

‡ Central Shops Department.

§ Electronics Division.

of particular interest, and are summarized in Table 37. From these data we may infer that the g threshold of morphological response is significantly less than an acceleration of 5×10^{-6} of constant sign. Determination of the g threshold to continuous acceleration, as well as the presentation-time characteristics of such acceleration, appears highly desirable. It is suggested that such threshold data are a necessary element in the programming of biosatellite accelerations.

Accordingly, a variable-velocity 2π compensator was developed. It can impose centrifugal forces from about $10^{-9} g$ to several g simultaneous with continuous compensation of earth g . Figure 90 shows the finished apparatus prepared for the rotation of eight test objects at a single radius. Figure 91 shows radial arms attached that enable simultaneous rotation of 40 test objects at five different radii.

The principle of the apparatus is as follows: Two concentric drive shafts are turned. One determines the centrifugal force applied to the biological objects by govern-

ing the rate of rotation of the spokes about the center hub. The second shaft rotates with a constant difference from the first, so that simultaneously the test objects are being constantly turned about the longitudinal axis of each spoke at 2 rpm to compensate for earth g . The system is powered by a two-phase synchronous hys-

TABLE 37
GROWTH OF ORGANS OF *Avena* SEEDLING AT
 $5 \times 10^{-6} \times g$ WHILE UNDER CONTINUOUS
COMPENSATION FOR EARTH g^*

	$+\Delta mm \pm \sigma_d$	"t"
Coleoptile. . . .	2.7 ± 0.3	$<0.01 - >0.001$
Mesocotyl. . . .	2.7 ± 0.7	$<0.05 - >0.02$
Shoot.	4.7 ± 1.0	$<0.02 - >0.01$
Root.	14.3 ± 4.8	$<0.09 - >0.05$

* Data taken at 70 hours after hydration, temperature 22°C . The values represent the greater length, in mm, of organs growing centrifugally over those growing centripetally; they are the means of three experiments, eight replicates in each. Chance probabilities are listed in the third column.

FIG. 89.—Two-dimensional compensator enabling a rotation of the test object at 2 rpm and at the same time applying a longitudinal force of $5 \times 10^{-6} \times g$.

FIG. 90

FIG. 91

FIGS. 90 AND 91.—Two-dimensional compensator enabling the 2 rpm rotation of the test object and, concomitantly, longitudinal accelerations from $3 \times g$ to $10^{-9} \times g$.

teresis drive developed here, using a variable frequency source to control angular velocity.

So far, one experiment has been run with the variable-velocity 2π compensator. The experimental acceleration was $5 \times 10^{-5} g$. The results qualitatively substantiate the indications of Table 37, i.e., growth rates under centrifugal orientation exceeded those under centripetal orientation. Experiments will continue, moving toward successively lower accelerations, to determine the response thresholds for continuous acceleration and to observe the sequelae of single, short-time exposures after initiation of growth.

We emphasize that organs developing on preexisting histological templates show no gross or microscopic changes in morphology when grown in the 2π compensator, though their growth and tropistic behavior are affected significantly. We have therefore become concerned with *de novo* development patterns under compensation, i.e., the differentiation of undifferentiated

studies are also detailed in accompanying reports (Miller and Gordon; Dedolph and Gordon).

4π

A major concern of this project is the development of apparatus to enable compensation in three dimensions—in the biological sense, to nullify uniformly the directional components of earth fields. To this end, a preliminary working model of a 4π compensator was developed from several pilot studies. Illustrated in Figure 93, it consisted of concentric gimbal rings pivoted at 90° , with primary power from the synchronous drive system. Each gimbal powered its successive neighbor by pulleys of fixed but unequal perimeter ratios. A plant, supported in a chamber within the inner gimbal, moved so that a point on its surface described diverse arcs distributed over the surface of a sphere. The distribution of orientations, as demonstrated by photography of a pin light simulating a point on the surface

FIG. 92.—Multiple unit clinostat for the rotation of test objects about a single axis

structures at both the zygote and somatic levels of organization. As a first step, we have initiated tissue cultures of tobacco pith, carrot phloem, and tomato roots. The culture requirements for *Arabidopsis* are described in the accompanying report by Miller, Sharp and Gordon. The parameters of differentiation are being determined for these materials, i.e., the concentration ratios of nutrient, auxin, and kinin that lead to predictable patterns of organ initiation, as well as those that keep parenchymatous tissues meristematic but undifferentiating.

1π

We have constructed 1π compensators that will permit screening experiments to be run on undifferentiated tissues. It is essentially the clinostat of classical plant physiology modified to enable numerous vessels to rotate, each on a single axis, at relatively low vibration levels. The apparatus is illustrated in Figure 92. It permits the rotation of each tissue about one central axis. These clinostats have also been used to determine the effect of single-axis gravitational compensation on geotropic and phototropic sensitivity. The results of these

of the biological object, appeared to yield a distribution of orientations that made initial biological trials worthwhile.

The apparatus proved unsuitable. Although it afforded a distribution of orientations of the test object of reasonable symmetry, the distributions of angular velocity and acceleration were sufficiently asymmetrical to produce a multiplicity of oriented force gradients well above perception thresholds.

Considerable time was spent in an empirical approach to better the pulley ratios between the gimbals. Various ratios were tested by photography of a pin light (representing a point on the surface of the organism) as the assembly underwent one period of rotation. The rate of return from this cut-and-try method was so slow and subjective that a more efficient analytical approach was indicated. The computer was programmed to determine the coordinates of the various orientations of the test object prescribed by any fixed pulley ratio between the gimbals. An oscilloscope was coupled to the computer to enable visualization, at timed intervals, of the orientations in space. In effect, the oscilloscope reproduced

the motion of the pin light described above. The orbits so obtained are illustrated in Figure 94 (gimbal velocity ratios 1:1:4). The dots shown represent successive orthogonal projections of a point on the test object. Their linear density and orientation represent the velocity and direction of movement of the test object. The orbit photographs provided a visual check here (and

will be employed similarly in the design discussed below).

The computer was then programmed to evaluate the combinations and permutations of fixed-pulley ratios in triplets from 1 to 1000. The criterion of acceptability was the magnitude of the Frobenius norm in first and then second spherical harmonics tests for the distribu-

FIG. 93.—Preliminary working model of a 3-dimensional compensator for multiple axes of rotation

FIG. 94.—Computer-derived positions in 3-dimensional space of a point on a biological object rotated in the apparatus shown in Fig. 93.

tions of orientation and angular velocity. Though numerous ratios passed the first spherical harmonics test, yielding Frobenius norms essentially at noise level, all fixed ratio combinations tested failed the second spherical harmonics test either in the distribution of orientation or the distribution of angular velocity. In short, we found that we could not achieve the experimental aim with apparatus using a fixed pulley ratio between concentric gimbals.

The problem may be defined as follows. Assume that the biological object is a sphere. The sphere is to be moved about its center, the center remaining fixed, so that a uniform distribution in space of sphere orientations, angular velocities, and angular accelerations is attained. The above requirements are not to take so long to be achieved that the organism is subject to tropistic stimulus.

A mathematically rigorous solution of the problem was achieved using analytical mechanics of rigid body motion; it will be presented by Cook elsewhere. In essence, our requirements can be obtained by basing the

orientation of motion on one of the five regular polytopes (Platonic solids) in a prescribed pattern. The icosahedron or dodecahedron yield satisfactory coverage of orientations. Mechanically, we needed to devise an apparatus that would rotate the biological test object through 60 positions of symmetry described, for example, by an icosahedron.

With the icosahedron there are 60 positions of symmetry possible; these positions are obtained by rotation of the polytope successively about paired vertices of each of the triangular faces. The 60 are achieved by three, almost identical, twenty-movement operations repeated for the three paired sets of vertices of any given polygonal face. However, if successive triangular faces are chosen for rotation at the end of each group of 60 movements, a total of 600 positions of symmetry is possible. These positions are identifiable with the 600 vertices of

the hecatonicosahedroid, a 4-dimensional polytope with 120 dodecahedral hyperfaces. By use of the more complex polytope, what we have done is to increase the degree of coverage of a sphere by a point on our biological object. We have done so economically, since the hardware required for the icosahedron solution is identical to that required for the hecaton derivative; all that is required is a relatively simple alteration in the programming operation.

The apparatus in the process of construction consists of the concentric gimbal system with which we began. However, each gimbal will be driven independently by a servo system. Computer-calculated coordinates for organism positions will be converted to analog form and taped. This information will be transduced through wave generators, and amplified to drive individually the three concentric gimbals containing the test organism.

GROWTH AND DEVELOPMENT IN COMPENSATED FIELDS: GROWTH AND GEOTROPIC CURVATURE OF OAT SEEDLINGS*

Richard R. Dedolph and Solon A. Gordon

PURPOSE AND METHODS

Geotropism response of plants requires both the magnitudes and directional components of the gravitational vector. If the directional component is nullified by clinostat rotation of the plant, no geotropic curvature occurs. This study was designed to determine the growth and geotropic sensitivity of seedlings grown so that the directional component of the gravitational field would be continuously nullified by clinostat rotation.

PROGRESS REPORT

Using hulled oat seeds (*Avena sativa* var. Victory) planted in moist quartz sand, it was found that coleoptiles of seedlings grown on a clinostat rotating at 2 rpm showed no curvature or directional growth trend not attributable to initial orientation of the seed. This showed that the directional component of the gravity vector was effectively nullified.

Clinostat-grown seedlings showed a greater capacity to respond to geostimulation, but no increase in minimum presentation time required for response demonstration (Figure 95).

Further studies of this increase in response capacity following geostimulation considered the effects of nullification of the directional vector component both before and after geostimulation, as well as the effects of rota-

FIG. 95.—The effect of nullification of the directional component of the gravitational vector in the pre- and poststimulation periods on response of *Avena* coleoptiles to varying periods of geostimulation.

* Work supported by the National Aeronautics and Space Administration.

tion *per se*. This was done by rotating plants about their longitudinal axis in a vertical position on devices identical to those used as clinostats. Within these rotated and within the clinostat-grown plants, all permutations of pre- and poststimulation conditions were used.

Data from these experiments (Table 38) showed that a part, but not all, of the increased response capacity to geostimulation of the clinostat-grown coleoptiles was due to physiological modification. A part of the difference was due to the nullification of the gravity counter-

TABLE 38

MEAN DEGREES CURVATURE* OF *Avena* COLEOPTILES EXPOSED TO VARIOUS PERIODS OF GEOSTIMULATION WITH DIFFERENT DEGREES OF NULLIFICATION† OF DIRECTIONAL COMPONENT OF GRAVITATIONAL VECTOR IN PRE- AND POSTPRESENTATION PERIODS

PREGEOSTIMULATION CONDITIONS	POSTGEOSTIMULATION CONDITIONS	MINUTES OF STATIONARY HORIZONTAL PRESENTATION		
		0	20	40
Clinostat rotated	Clinostat rotated	-1.00 _i	19.12 _b	31.08 _a
Stationary	Clinostat rotated	-0.05 _i	10.24 _{efgh}	16.08 _{bc}
Clinostat rotated	Stationary	0.15 _i	15.42 _{bed}	17.35 _b
Stationary	Stationary	0.25 _i	7.60 _{gh}	11.38 _{efg}
Vertically rotated	Vertically rotated	0.05 _i	7.35 _h	12.55 _{cde}
Stationary	Vertically rotated	0.02 _i	7.30 _h	12.35 _{de}
Vertically rotated	Stationary	-0.12 _i	7.90 _{fgh}	11.72 _{def}
Stationary	Stationary	0.00 _i	7.73 _{gh}	11.20 _{efgh}

* Means not followed by a common subscript differ at odds of 99:1.

† Clinostat rotation as employed resulted in complete nullification of the directional component. Vertical rotation employed imparted all of the rotational system characteristics other than directional component nullification.

stimulation during the one-hour curvature development period following geostimulation.

Suggestions as to the nature of these physiological modifications are afforded by growth and germination data (Table 39). Emphasis was placed on the comparison of vertically rotated and clinostat-grown seedlings because previous experiments showed that these two conditions had produced seedlings of like but greater coleoptile weights than either of the stationary controls. Root growth of the seedlings grown with vertical rotation did not differ from the stationary controls but was less than that of clinostat-grown seedlings. The different geotropic response between clinostat and vertically rotated coleoptiles was not, therefore, explicable on the basis of difference in average weights or lengths.

TABLE 39

EFFECTS OF NULLIFICATION OF DIRECTIONAL COMPONENT OF GRAVITATIONAL VECTOR ON GROWTH AND DEVELOPMENT OF OAT SEEDLINGS HARVESTED 72-82.5 HOURS AFTER INITIAL SOAKING

GROWTH CONDITION	PER CENT GERMINATION*	COLEOPTILES*		ROOTS PER PLANT*		
		Fr. wt., mg	Length, mm	No.	Fr. wt., mg	Length, mm
Clinostat rotated..	68.7 _a	33.6 _a	27.8 _a	4.1 _a	47.2 _a	36.1 _a
Vertically rotated..	68.1 _a	30.4 _a	25.8 _a	4.1 _a	39.0 _b	25.4 _b
Number of seed or experimental cell averages used in determining each mean.....	160	40	40	40	40	40

* Means within a column followed by the same subscript do not differ at odds of 19:1. Means within a column followed by different subscripts differ at odds of 99:1.

GEOELECTRIC EFFECT AND GEOTROPIC CURVATURE*

Richard R. Dedolph, Joseph J. Breen, and Solon A. Gordon

PURPOSE AND METHODS

Shoot and root tips of many plant species will develop electrical potentials between the upper and lower sides following reorientation from a vertical to a horizontal position. This phenomenon is known as the geoelectric effect. Contemporary^(1, 2) as well as older studies⁽³⁻⁷⁾ have sought to relate this phenomenon causally to a lateral redistribution of the growth hormone, auxin, and thence to geotropic curvature. Recently it was concluded that the geoelectric effect is a secondary consequence of the asymmetric distribution of

* Work supported by the National Aeronautics and Space Administration.

auxin, and that the geoelectric hypothesis of geotropism is incorrect.⁽⁸⁾

The above refutation was based on an inversion of the cause and effect roles of the geoelectric effect and auxin distribution; it was shown⁽⁸⁾ that auxin is essential for the development of the potential. Both the geoelectric hypothesis and its subsequent refutation assume that the geoelectric effect and geotropic curvature are related through an asymmetry of auxin distribution. The possibility that the geoelectric effect and geotropic curvature might in fact not be related remained untested. We have, therefore, experimentally tested this possibility.

Intact corn (*Zea mays* cv Hybrid 64a × 22R; University of Wisconsin) coleoptiles were grown at 25° C on tap-water-soaked blotting paper under red light (G.E. Ruby Red). After four days, when the coleoptiles were 30–40 mm in length, they were transferred to an air-tight glove box. Through the box passed a constant flow of water-saturated air or nitrogen. The flow rate of the nitrogen used to establish anaerobic conditions was such that the oxygen concentration of the chamber was reduced by dilution to less than 0.01% before geotropic stimulation was applied. A corresponding flow rate of air was employed when coleoptiles were held under aerobic

through a calomel half-cell to an electrometer (input impedance 10^8 ohms). After an additional hour of equilibration with the electrode contact established, determinations of the transverse potential were begun. Measurements were made every 5 minutes for 4 hours when the gas employed was air, and every 20 minutes for 8 hours when the gas was nitrogen.

One hour after the initial potential measurement, the plants were all tipped to a horizontal position. Plants under air were returned to a vertical position after one hour of this stimulation. Plants under nitrogen received four hours of horizontal stimulation. At the end of the

FIG. 96.—The transverse geoelectric potential of *Zea mays* coleoptiles under aerobic and anaerobic conditions

conditions. The gas exhaust was so adjusted that the box was under a slight positive pressure with respect to the surrounding atmosphere during the experiment. The temperature within the box was about 26.5° C, 1.5° higher than in that area of the surrounding room in which the coleoptiles were grown.

Following transfer to the box all illumination other than phototropically inactive safe-lights was discontinued. One plant was placed vertically in an electrode holder and positioning assembly. Eight plants were placed adjacent to it in a similar vertical position on the bottom of the box. After 3 hours of equilibration, electrode contact was established with the coleoptile by extruding saturated KCl solution in 1.5% agar from the tip of the electrode assembly. Contact was made 1 cm below the tip on diametrically opposite sides of the coleoptile. These agar-KCl gel contacts were linked

stimulation period, plants not used for potential measurement were shadowgraphed to determine degrees curvature, i.e., the angle between a line projected through the central axis of the tip and a line projected through the central axis of the base of the coleoptile. Replication within anaerobic and aerobic conditions was attained by repetition of these treatments and procedures using different seedlings. Average standard deviations and 95% confidence intervals of means for each gas treatment were determined from pooled estimates of error variance.

PROGRESS REPORT

The geoelectric potential data thus obtained (Figure 96) almost exactly reproduced published data of others^(1, 8) obtained by methods not involving direct electrode contact of coleoptiles. All of these data show

that no geoelectric potential is developed under anoxia.

However, coleoptiles showed negative geotropic curvature regardless of the gaseous atmosphere surrounding them. Those under anaerobic conditions were curved 17 ± 1 degrees after 4 hours of stimulation. After 1 hour of stimulation those under aerobic conditions curved 18 ± 1 degrees.

In the determination of geoelectric potentials by a method not involving direct contact with the coleoptile tip,⁽⁸⁾ it was established that the removal of coleoptile tips virtually eliminated curvature response to geotropic stimulation without modifying materially the potentials produced by geostimulation. This elimination demonstrates that the geoelectric effect occurs in the absence of geotropic curvature. We observe that curvature develops under anoxia, a condition under which no detectable potential gradient develops. In other words, geotropism occurs in the absence of the geoelectric effect.

These facts demonstrate the lack of causal or direct relationship between the geotropic and geoelectric effects. Auxin is undoubtedly required for both the tropic

and electrical expression of the gravitational stimulus. But the asymmetry of the hormone could well be a consequence of a direct action of the gravity sensor either on the reactivity to auxin of responsive cells, or on their ability to translocate auxin basipetally.

REFERENCES

1. Grahm, L. and C. H. Hertz. Measurements of the geoelectric effect in coleoptiles by a new technique. *Physiol. Plantarum* 15, 96-114 (1962).
2. Grahm L. and C. H. Hertz. Measurements of the geoelectric effect in coleoptiles. *Physiol. Plantarum* 17, 186-201 (1964).
3. Amlong, H. U. Untersuchungen über die Beziehungen zwischen geoelektrischen Effekt und Geotropismus. *Planta (Berl.)* 21, 211-250 (1933).
4. Cholodny, N. Wuchshormone und Tropismen bei der Pflanzen. *Biol. Zbl.* 47, 604-626 (1927).
5. Dolk, H. E. Geotropie en Groeistoff. Diss. Utrecht (1930).
6. Schrank, A. R. Analysis of the effects of gravity on the electric correlation field in the coleoptile of *Avena sativa* *Bioelectric Fields and Growth*, ed. E. J. Lund. University of Texas Press, Austin, 1947, pp. 75-123.
7. Went, F. W. Eine botanische Polaritätstheorie. *Jahrb. wiss. Bot.* 76, 528-557 (1932).
8. Grahm, Lennart. Measurements of geoelectric and auxin-induced potentials in coleoptiles with a refined vibrating electrode technique. *Physiol. Plantarum* 17, 231-261 (1964).

GROWTH AND DEVELOPMENT IN COMPENSATED FIELDS: PHOTOTROPIC SENSITIVITY AND GROWTH OF AVENA*

Jane Shen Miller, Martha L. Preston, and Solon A. Gordon

PURPOSE AND METHODS

This study was designed to determine if subjecting a plant to continuous gravitational compensation gives rise to a change in growth rate and phototropic sensitivity.

Avena seeds (Svalöf Victory I) were hydrated prior to planting on agar (1%) medium. The medium was poured in a 150-ml Erlenmeyer flask whose top was cut off at ca. 3.5 cm from the base. This was done to facilitate phototropic exposure of the seedling without interference by the glass. Four seeds were planted to a flask, each at an angle of 45° to the agar surface, to obtain a coleoptile orientation normal to the agar surface. Only one seedling was selected from each flask. The top and the base of the flask were sealed with masking tape, and neck stoppered by a cotton plug. The assembly was placed in a single-axis clinostat designed to hold ten such flasks; the longitudinal axis of the seedling was orientated horizontally on the axis of rotation. The speed of rotation was 2 rpm. A factorial design was used for experimental treatments:

- C-C: compensation during growth and curvature development;
- C-NC: compensation during growth but not during curvature development;
- NC-C: no compensation during growth but during curvature development;
- NC-NC: no compensation during growth or curvature development.

Red light was given from the day of seeding for a period of 24 hours. The plants were then grown in complete darkness except that a green safe-light was used for experimental manipulation. Exposure to phototropic stimulation took place ca. 70 hours after planting. A tungsten filament lamp with a blue glass filter (Corning 5-59) was used as the light source. Two hours following irradiation, shadowgraphs were made of the coleoptiles; their curvatures were measured subsequently with a goniometer.

PROGRESS REPORT

A dose-response curve was determined for each treatment in the above factorial design, using unilateral irradiances of ca. 3 to 13 kiloergs cm^{-2} , exposure time 15 seconds. Figure 97 and Table 40 show the responses, each datum representing an average of 70 measure-

* Work supported by the National Aeronautics and Space Administration.

ments. The optimum exposure for all treatments was about 9 to 10 kiloergs cm^{-2} . Treatment NC-C elicited the greatest curvature, followed, in order, by C-C, NC-NC, and C-NC. Compensation during growth reduces the phototropic sensitivity of the seedling, but if applied subsequent to phototropic stimulation, enhances curvature development. All of the compensation treatments differ from one another at a significance level of $<1\%$ (F and Studentized Multiple Range tests).

Length and fresh weight measurements of noncompensated (NC) and compensated (C) *Avena* seedlings were made on the coleoptile, mesocotyl, and roots. They are tabulated in Table 41. Except for the mesocotyl, in all instances the organs of the compensated seedling were significantly retarded in growth when compared to those noncompensated. The ratios of NC to C in length

FIG. 97.—Curvature response of *Avena* coleoptiles to phototropic stimulations as affected by gravitational compensation; C-C, compensation during growth and curvature development; NC-C, no compensation during growth, but during curvature development; C-NC, compensation during growth but not during curvature development; NC-NC, no compensation during growth or curvature development.

and weight measurements are 1.4 and 1.7, respectively, for the organs that showed significant differences, i.e., the root and coleoptile respond similarly to compensation.

Continuous compensation of *Avena* during growth reduces the response of the coleoptile to phototropic stimulation. This may be related to the growth rate of this organ, since the growth of both the coleoptile and root in compensated plants was less than that of those noncompensated. However, compensation given to plants

following phototropic stimulation enhances curvature development. The curvature of a noncompensated coleoptile is the product of two opposing forces, light and gravity. Consequently, the net curvature is less than that of a coleoptile compensated for gravity following irradiation. Comparing treatment differences in Table 40, we find that 1) compensation during growth

TABLE 40

EFFECT OF GRAVITATIONAL COMPENSATION ON CURVATURE RESPONSE OF *Avena* COLEOPTILE TO PHOTOTROPIC STIMULATION

Compensation, kiloergs cm^{-2}	NC-C, Deg	C-C, Deg	NC-NC, Deg	C-NC, Deg	Avg., Deg
3.6	22.0	18.7	9.5	8.2	14.6 _x
6.4	29.2	24.4	15.6	8.2	19.4 _x
7.8	37.0	32.0	17.9	13.3	25.0 _y
9.4	40.9	30.4	23.9	17.4	28.2 _y
13.2	25.9	29.7	17.3	11.8	21.2 _x
Avg.	31.0 _a	27.0 _b	16.8 _c	11.8 _d	21.7

A common letter indicates a difference not significant at the 1% level.

TABLE 41

GROWTH OF NONCOMPENSATED (NC) AND COMPENSATED (C) *Avena* SEEDLINGS (70 HR, 25° C)

	COLEOPTILE		MESOCOTYL		SHOOT		ROOT	
	mm	mg	mm	mg	mm	mg	mm	mg
NC.....	16.2	20.9	4.2	7.6	20.8	28.4	127.6	36.6
C.....	11.3	11.6	4.4	6.4	15.6	18.2	92.1	20.9
NC/C.....	1.4	1.8	0.95	1.2	1.4	1.6	1.4	1.7

The differences between NC and C for all organs except the mesocotyl are significant at the 1% level.

reduces phototropic curvature by ca. 4.5°; 2) compensation following irradiation enhances curvature by 14.2°; 3) comparing C-C with NC-NC, there is a difference of 10.2° which is similar in magnitude to the difference of 1) and 2); and 4) comparing NC-C with C-NC, the difference is approximately the sum of 1) and 2). These comparisons indicate that compensation after irradiation has a greater effect on phototropic curvature than does compensation during the growth period before phototropic stimulation.

In summary we may state that, under the conditions described, growth of plants under compensation reduces their phototropic responsivity. This reduction could result from 1) a lessening of hormonal imbalance induced by unilateral irradiation, 2) a lowering of the reactivity of the tissues to the hormonal imbalance so induced, 3) a decrease in hormonal titer, resulting in a change in kinetics of the curvature response, 4) a decrease in the velocity of hormone transport.

AN IMPROVED MEDIUM FOR RAPID INITIATION OF ARABIDOPSIS TISSUE CULTURE FROM SEED*

Jane Shen Miller, William R. Sharp, and Solon A. Gordon

PURPOSE AND METHODS

Arabidopsis thaliana is a small, rapidly growing crucifer with a 28-day life cycle, a low chromosome number, $n = 5$, and a prolific seed production. Its small size and short cycle make it highly suitable for studies of gravity compensation, particularly with equipment for rotation in three dimensions. The present study was undertaken to determine the requirements for *in vitro* culture of this organism.

We have observed that *Arabidopsis* can complete its life cycle under aseptic conditions in an agar medium, provided the trace elements are chelated, roots are shielded from light, gaseous exchange is adequate, and the temperature within the growth container is not above 25° C. Higher temperatures and poor aeration resulted in the formation of aborted embryos.

PROGRESS REPORT

To study *de novo* morphogenesis of *Arabidopsis* under compensation, cultures of undifferentiated totipotent cell masses were required. In preliminary trials, seeds were planted on a modified White's medium⁽¹⁾ containing 20% coconut milk and 1.5% agar. Seeds germinated on the third day with the emergence of two small leaves. The leaves did not expand, but remained green for several weeks. Undifferentiated tissue proliferated from the seed 1.5 months after seeding. The leaves became

* Work supported by the National Aeronautics and Space Administration.

etiolated as this tissue developed. Such etiolation occurs under both tungsten-filament bulb illumination at ca. 15 fc and mixed incandescent-fluorescent tube illumination at 2000 fc. Subsequent growth of the tissue was also extremely slow on the above medium.

A medium was, therefore, developed to obtain rapid initiation of callus from seed. It consists of a supplemented White's standard nutrient agar.⁽²⁾ Good callus formation was observed within two weeks after planting of the seed. All of the seed developed callus within 3 weeks. The supplement contains 10 mg of each of the following compounds per liter of water: naphthaleneacetic acid, 2,4-dichlorophenoxyacetic acid, kinetin, gibberellic acid, inositol, biotin, folic acid, choline, and pantothenic acid. This solution is sterilized by Millipore filtration and kept in frozen storage. One hundred ml of the supplement solution is added to each liter of White's nutrient agar for the initiation of callus tissue.

After the callus is established, it is transferred to a tobacco culture medium⁽³⁾ supplemented by 2 mg of indoleacetic acid and 0.64 mg kinetin per liter of medium for rapid subsequent growth. We were unable to initiate the *Arabidopsis* callus on this tobacco medium.

REFERENCES

1. Lamport, D. T. A. Oxygen fixation into hydroxyproline of plant cell wall protein. *J. Biol. Chem.* **238**, 1438-1440 (1963).
2. White, P. R. The cultivation of animal and plant cells. The Ronald Press Co., New York, 1963, 2nd ed., p. 60.
3. Murashige, T. and F. Skoog. A revised medium for rapid growth and bioassay with tobacco tissue culture. *Physiol. Plantarum* **15**, 473-497 (1962).

HORMONAL RELATIONS IN THE PHOTOTROPIC RESPONSE II. TRANSLOCATION OF C¹⁴-LABELED INDOLEACETIC ACID IN IRRADIATED AVENA COLEOPTILE SEGMENTS

Solon A. Gordon and Marcia Eib

PURPOSE AND METHODS

According to the Cholodny-Went theory of tropism, transverse polarization is induced in receptor tissues by unilateral irradiation. A lateral transport of auxin takes place, resulting in an unequal distribution of the hormone. This inequality is transported basipetally, eliciting a corresponding difference in the growth rates of tissues subjacent to the receptor region, and causing

a bending of the shoot in the plane of the light direction. Essential to this theory is a lateral translocation of the hormone, an active hormonal-transport system, and a basipetal polarity of such transport. In the first paper of this series,⁽¹⁾ using C¹⁴-labeled indoleacetic acid (IAA), we reaffirmed the existence of auxin transport rates consistent with the kinetics of the tropic response, and the strict basipolarity of the transport. The present

report deals with lateral movement of the labeled hormone in tissues of the coleoptile tip exposed to phototropic stimulus.

C^{14} -IAA, methylene labeled with a specific activity of 12.6 c/M, was synthesized in this laboratory.⁽²⁾ The auxin was purified from radioactive decomposition products by paper chromatography⁽³⁾ for each experimental series. Seed of *Avena sativa* var. Victory (Svalöf) were exposed to red light (G.E. Ruby Red, $7\frac{1}{2}$ watts) for 24 hours after soaking. They were then grown in the dark in closed moist chambers. Seventy-two hours after soaking, 3-mm tips, or 5-mm segments cut 3-mm from the tip were taken for experiment. The tissues were kept

were dried on planchets. Counting was done with a windowless flow counter in the proportional region, correcting for self-absorption where required.

The light source for phototropic stimulation was a tungsten-filament projection lamp with a Corning 5-59 Projection Blue filter. This yielded an incident irradiance, at 1.3 m distance, of $10 \mu W cm^{-2}$. For equilateral irradiation, the beam of light was reflected downward by a mirror mounted at an angle 45° from the horizontal directly above the tips. Radiant densities of $300 \mu joules cm^{-2}$ were used throughout for stimulation. This energy elicits maximum first positive curvature when given unilaterally (Figure 98).

FIG. 98.—Exposure-response curve for phototropic stimulation

constantly in normal vertical orientation to preclude geotropic stimulation. For the introduction of the labeled auxin, unless otherwise noted, the tips were placed on the sharpened teeth of small combs; the assemblies were then immersed several minutes in an aqueous solution of the auxin at a concentration of $10 \mu g/ml$. They were then rinsed with distilled water and irradiated with blue light (see below).

Immediately after irradiation, the tissues were mounted vertically on a stainless steel razor blade so that the sharp edge penetrated about 1 mm into the base of the tissue, which in turn pressed against a receiver block of 1.5% agar. The blade was supported by a block clamp, and aligned to bisect the long transverse axis of the coleoptile. (All metallic surfaces were previously coated with silicone oil.)

For the determination of radioactivity agar blocks were placed in lipped aluminum planchets, covered with water to permit the auxin to distribute evenly, and then dried. Tissues were dispersed in water with a Potter-Elvehjem homogenizer; aliquots of the dispersion

PROGRESS REPORT

Experiment I

Does lateral displacement of auxin occur after equilateral irradiation of coleoptile tissues without tip? Subapical coleoptile segments were mounted on agar blocks bisected by the razor blade as shown in Figure 99. Auxin was supplied to the apical end of the coleoptile cylinder by a donor block. Assemblies were irradiated equilaterally and then permitted to remain for three hours, in the dark, in a moist chamber. The receiver blocks were then removed for counting. The results, given in Figure 99, show that no loss in radioactivity occurred as a result of exposure to light, and that equilateral irradiation of subapical coleoptile tissues does not cause lateral inequalities in the auxin basipetally transported through the tissues.

Experiment II

Since the coleoptile tip is the region of the organ where lateral movement presumably takes place, the preceding experiment was repeated with coleoptile tips

instead of coleoptile sections. The results are given in Figure 100. Equilateral irradiation of the tip neither changes the amount of auxin translocated basipetally nor affects significantly the lateral equality of that transport.

Experiment III

The influence of unilateral irradiation of the tip upon auxin movement was then determined. Tips presoaked in the labeled auxin solution were irradiated and then placed on the agar blocks as shown in Figure 101. Unilateral irradiation did not change significantly the equality in the amounts of auxin diffusing from the proximal and distal sides. Though the splitting of the base by the razor blade did not lower the total activity measured, significantly more auxin was transported from the tissues into the blocks when the blade divided the system.

FIG. 99.—Effect of equilateral irradiation of coleoptile sections on the amount and lateral distribution of basipetally translocated auxin. Left: $n = 1$, 73 replicates. Right: $n = 1$, 77 replicates.

FIG. 100.—Effect of equilateral irradiation of the coleoptile tip on the lateral distribution of auxin diffusing from their bases. Left: $n = 2$; 110 and 73 replicates. Right: $n = 3$; 107, 75, and 72 replicates.

Experiment IV

The preceding experiments were based on activities diffusing from the tip into the receiver blocks. We then asked whether unilateral irradiation would change the auxin distribution if extraction of the tissues was used to measure the auxin in the tissues proximal and distal to the light source. Infiltrated coleoptile tips were mounted on razor blades so that about 1 mm of the blade was in the tissue (Figure 102). The tips were irradiated unilaterally and then allowed to remain *in situ* for 90 minutes. They were then pushed downward on the blade so that the tissues were slit into their proximal and distal halves. The data in Figure 102 demonstrate no difference in lateral distribution of activity and no change in the total activity following unilateral irradiation.

Experiment V

No resolution of the extracts or diffusates was done before assay in the previous experiments. The radioactivity was considered to emanate solely from IAA, since in previous work⁽¹⁾ only one radioactive band was found

FIG. 101.—The transport of labeled auxin from unilaterally irradiated tips. Left: $n = 1$, 69 replicates. Right: $n = 1$, 80 replicates.

FIG. 102.—The effect of unilateral irradiation on the amount of activity extracted from the proximal and distal tissues. Left: $n = 1$, 139 replicates. Right: $n = 1$, 125 replicates.

on chromatography of the diffusate, a band with the Rf of synthetic IAA. To test the inferences from Experiments I to IV more rigorously, chromatographic resolution was incorporated before assay.

Tips were irradiated unilaterally according to the protocol outlined in Table 42. After two hours of diffusion there was no significant difference in the direct counts of proximal and distal receiver blocks. The same blocks were then extracted with ether and the extract chromatographed. Assay of the IAA area in a strip counter again showed no significant difference in radioactivity attributable to IAA. However, in a parallel experiment that did not involve the introduction of labeled IAA into the tissues before unilateral irradiation, assay of the endogenous IAA areas on the chromatogram by the *Avena* section test showed an unequal concentration of auxin in the blocks. The proximal blocks, i.e., the blocks under the "light" tissues, had ca. 43% of the total auxin, whereas the distal blocks, i.e., those under the "dark" tissues, contained about 57% of the auxin. These differences are significant at the 1% level, though the ratio is smaller than that observed by others in proximal and distal diffusates. No biological activity was detected in any region of the chromatogram other than that at the Rf of IAA.

The results so far may be summarized as follows. The auxin basipetally transported appears to be IAA, and both endogenous and exogenously introduced IAA are not destroyed by irradiation. Introduced IAA is apparently not transported laterally following equilateral or unilateral irradiation. But endogenous IAA apparently does move transversely upon unilateral irradiation, judging from the concentration differences in the diffusates from the proximal and distal tissues.

What, then, explains the differences observed in the behavior of endogenous and exogenously-supplied IAA?

1. Perhaps the native auxin moving basipetally as a hormone is not IAA. This seems highly improbable. Numerous direct examinations⁽⁴⁻⁸⁾ as well as less direct evidence⁽⁹⁾ indicate IAA to be native to the grass seedling and functioning as a longitudinal correlative agent.

2. Light could cause the conversion of IAA to a C¹⁴-labeled product possessing the same chromatographic properties as IAA but of lower activity as an auxin. This could result in transversely equal radioactivities when C¹⁴-labeled IAA was supplied, but unequal physiological activities. A model for such a product may be inferred from studies⁽¹⁰⁾ on the *in vitro* riboflavin-sensitized photolysis of IAA. The pathway of this photooxidation goes from IAA to indolealdehyde through indoleglycolic and indoleglyoxylic acids. Indoleglyoxylic acid has virtually the same Rf as IAA in a number of solvent systems, but its auxin activity is low.

We believe that such decomposition mediated by

light is not relevant for the following two reasons. First, the data in Table 42 on the biological activity at the IAA locus on the chromatograms show no significant reduction in total activity after irradiation. The work of Briggs et al.⁽¹¹⁾ more rigorously demonstrates that no loss in total biological activity follows irradiation. In their experiments the *Avena* curvature test was used as the assay; the curvature test is more specific for IAA in that it depends on the relatively unique basipetal transport characteristics of this auxin.

Second, the migration rate of the radioactivity upon chromatography is unaffected by treatment with a carbonyl reagent. In an experiment similar to that outlined in Table 42, the ether extract of the blocks was treated with 2,4-dinitrophenylhydrazine before chromatography. No difference in radioactivity of the IAA

TABLE 42

EFFECT OF UNILATERAL IRRADIATION ON AUXIN DISTRIBUTION IN RECEIVER BLOCKS WHEN CHROMATOGRAPHIC RESOLUTION WAS INCORPORATED BEFORE ASSAY

	Distal ("Dark")	Proximal ("Light")	Conditions
Exogenous C ¹⁴ -IAA, cpm.....	21.5 ± 2.3 87 ± 3	22.2 ± 2.9 91 ± 5	Direct counting of blocks IAA area on chro- matogram of block extract
Endogenous IAA, µg × 10 ²	15.4 ± 0.62	11.8 ± 0.81	IAA area on chro- matogram bioas- sayed by the <i>Avena</i> section test

areas on the chromatogram was observed, comparing the proximal and distal diffusates from unilaterally irradiated or unirradiated coleoptiles. It may be inferred that no carbonylic decomposition product of IAA, with a similar migration rate, diffuses from phototropically stimulated coleoptile tips.

3. The necessity for invoking a lateral transport of auxin can be avoided by postulating an action of light on auxin biogenesis. Assume that the rate of auxin production in the tip of the organ is substrate limited. A light-induced inhibition of the enzymatic process on the proximal side might permit unutilized precursor to move laterally to the dark side, and be there converted. The total amount of auxin produced would remain the same.

As has been described in detail elsewhere,⁽⁸⁾ auxin biogenesis is localized in the coleoptile tip. Precursors of auxin are produced in the germinating seed, translocated to the shoot apex, and are there converted to auxin. The biochemistry of auxin formation is not yet precisely

defined, and the identity of the intermediate translocated from the seed to the shoot apex is conjectural. Nevertheless, a strong case can be made for the thesis that tryptophan is the primary precursor of IAA, and that the endosperm of cotyledon is the source of auxin precursor for the shoot of a germinating seed. Accordingly, we determined the effect of unilateral irradiation on the capacity of tip tissues to form auxin from both tryptophan and seed substrates. The capacity was tested under conditions of substrate saturation, i.e., zero order reaction rates.

Experiment VI

Coleoptile tips were irradiated unilaterally (procedure of Experiment IV) and split immediately following exposure into proximal and distal halves. In one

TABLE 43
AUXIN FORMATION BY *Avena* COLEOPTILE TIPS
FOLLOWING UNILATERAL IRRADIATION

Tissue	Substrate	M μ g Auxin*/ 20 Tips/Hr	
"Intact" tips	Tryptophan	Light 1/2.....	14
		Dark 1/2.....	13
	Seed extract	Light 1/2.....	12
		Dark 1/2.....	15
Cytolyzed tips	Tryptophan	Light 1/2.....	44
		Dark 1/2.....	38
	Seed extract	Light 1/2.....	26
		Dark 1/2.....	25

* IAA equivalents by *Avena* curvature test.

series the halves were dropped directly into 0.04 M L-tryptophan, pH 7.2, or an aqueous extract of *Avena* seed, pH 7.2. The extract was prepared by germinating *Avena* seed one day at 25° under sterile conditions, dispersing the seed in 3 times its weight of 0.5% NaCl, and clarifying the dispersion by centrifugation at 110,000 $\times$ g for 15 minutes. The incubation was carried out at 30° in the dark. At half-hour intervals, an aliquot was taken and fractionated to obtain the acidic components. These were assayed for auxin activity by the *Avena* curvature test (cf. Reference 12 for details). In a comparable series, the half-tips were cytolized by liquid nitrogen before substrate solution was poured on them. The effects of the various treatments on auxin production are shown in Table 43, and represent rates fitted by least squares. The cytolized tips were more effective than the "intact" tips in converting both tryptophan and seed extract precursors to auxin. However, in no instance

were the yields from the light and dark tissues different from one another at the 10% level of significance.

The region of the coleoptile most sensitive to phototropic stimulation is the apical 0.1–0.2 mm. It could be argued that lateral transport of IAA is relatively restricted to this point. Thus, if the labeled auxin is introduced into an entire 3-mm tip, the amount of auxin actually moving laterally in the uppermost region after stimulation would be a small fraction of the total auxin content. Lateral movement could be thereby masked. In the following experiment, therefore, IAA was supplied only to the most apical portion of the tip.

Experiment VII

Labeled IAA was taken up in hydrated lanolin (final concentration 350 μ g/g). Coleoptile tips were mounted on agar blocks bisected by razor blades, as in Figure 100. Under magnifying spectacles, using a fine wire spatula, a small amount of the lanolin was applied symmetrically to the uppermost portion of the coleoptile tip. The assemblies were irradiated unilaterally and then transferred to a moist chamber at 25°. Two hours after irradiation the uppermost tissue on which the lanolin rested was sliced off and discarded. The remainder was split by pushing it downward on the blade, yielding proximal and distal halves. Half-tissues and blocks were pooled and dispersed for counting. The proximal ("light") side yielded an average count per assembly of 64 ± 4 cpm; the average of the distal ("dark") side was 59 ± 3 cpm. We conclude from this absence of significant imbalance that no lateral translocation of the labeled auxin was induced by unilateral irradiation.

In summary, we find that C¹⁴-labeled IAA introduced into apical or subapical tissues of the coleoptile is neither destroyed nor translocated laterally following either equilateral or unilateral irradiances with energies that elicit "first-positive" curvatures. However, inequalities of endogenous IAA are translocated basipetally following unilateral irradiation. The apparent differences in behavior of endogenous and exogenous IAA cannot be explained by a photolysis of the latter. Neither does it appear that an imbalance of auxin biosynthesis is induced by unilateral irradiation. (It is possible, of course, that the substrates used in the biosynthesis experiments, tryptophan and seed extract, may not be those normally functional in the coleoptile tip.) That IAA *per se* is not laterally translocated is further supported by the observed lack of imbalance when auxin was applied directly and specifically to the most photoreceptive region of the coleoptile, the apex.

Since there are numerous studies which show that a mechanical barrier inserted normal to the light path into the tip impairs the phototropic response, a material

factor must move laterally after phototropic stimulation. We suggest therefore the following alternatives. First, some complex of IAA, not formed from exogenously supplied auxin, is laterally translocated across the tip after unilateral irradiation, and is subsequently converted to IAA for basipolar transport. Second, a material factor other than IAA moves laterally after stimulation, and influences the rate at which the hormone is basipetally translocated. Either of these explanations would be compatible with the lack of effect of the stimulus on the total IAA level. Both would characterize the phototropic response as a phenomenon mediated by two hormones, rather than one.

REFERENCES

1. Gordon, S. A. and M. Eib. Hormonal relations in the phototropic response. I. Polarity and velocity of auxin transport. Argonne National Laboratory Quarterly Report of the Biological and Medical Research Division. ANL-5597, p. 51; also, *Plant Physiol.* **31**, XIV (1956).
2. Stutz, R. E., D. E. Atkinson and S. A. Gordon. Synthesis of 3-indoleacetic acid-2-C¹⁴, Argonne National Laboratory Report. ANL-4710 (1951).
3. Gordon, S. A. and L. Paleg. Observations on the quantitative determination of indoleacetic acid. *Physiol. Plantarum* **10**, 39-47 (1957).
4. Wildman, S. G. and J. Bonner. Observations on the chemical nature and formation of auxin in the *Avena* coleoptile. *Am. J. Bot.* **35**, 740-746, (1948).
5. Reinert, J. Über den Wuchsstoffgehalt der *Avena*-Koleoptilspitze und die chemische Natur des extrahierbaren Auxins. *Z. Naturforsch.* **5B**, 374-380 (1950).
6. Terpstra, W. Chromatographic identification of the growth substance extracted from *Avena* coleoptile tips. *Koninkl. Med. Akad. Wetensch. Proc.* **56**, 206-213 (1953).
7. Söding, H. and E. Raadts. Chematographische Untersuchungen über die Wuchsstoffe und Hemmstoffe der Haferkoleoptile. *The Chemistry and Mode of Action of Plant Substances*, ed. R. L. Wain and F. Wightman. Butterworth, London, 1956, pp. 52-56.
8. Shibaoka, H. and T. Yamaki. A sensitized *Avena* curvature test and identification of the diffusible auxin in *Avena* coleoptile. *Bot. Mag. Tokyo* **72**, (850), 152-159 (1959).
9. Gordon, S. A. The biogenesis of auxin. *Handb. d. Pflanzenphysiologie, XIV*, eds. W. Ruhland and H. Burström. Springer-Berlin, 1961.
10. Fischer, A. Über die papierchromatographische und papier-elektrophoretische Trennung von Indolderivaten. *Planta* **43**, 288-314 (1954).
11. Briggs, R. W., R. D. Tocher and J. F. Wilson. Phototropic auxin redistribution in corn coleoptiles. *Science* **126**, 210-212 (1957).
12. Gordon, S. A. and E. Buess. Inactivity of hexitols and hexoses in biosynthesis of auxin from tryptophan. *Plant Physiol.* **38**, 11 (1963).

HORMONAL RELATIONS IN THE PHOTOTROPIC RESPONSE. III. LIGHT-INDUCED CHANGES OF ENDOGENOUS AUXINS IN THE COLEOPTILE

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PURPOSE AND METHODS

According to the Cholodny-Went theory, phototropism in plants is caused by a lateral migration of the hormone auxin from the irradiated to the shaded side of an organ upon unilateral irradiation. As a result, unequal rates of longitudinal growth occur in the laterally adjacent tissues. Our previous studies with exogenously applied C¹⁴-labeled indoleacetic acid (auxin)⁽¹⁾ indicate, however, that lateral inequality in auxin distribution was not found in phototropically stimulated coleoptiles, and that two hormones may be involved in the phototropic response. The present investigation, therefore, undertook to reexamine the hormonal changes induced by light.

Coleoptiles were obtained from seeds of *Avena sativa* (Svalöf Victory I) and *Zea mays* (Snowcross). For irradiation, 5-mm tips were cut and aligned on a moistened microscope slide. Unilateral irradiations were performed only with tips of *Zea*. Groups of 15 tips were mounted vertically on a stainless steel razor blade, 3 × 35 mm, so that the sharp edge penetrated ca. 1 mm into the base of the tip. The tips were split into irradiated and shaded halves after irradiation.

Manipulations were carried out either under red light or light from green fluorescent tubes filtered through green and amber Plexiglas.

Since action spectra for first positive phototropism show a major band in the region of 450 mμ wavelength,⁽²⁾ blue light was used for phototropic stimulation of the coleoptile tips. Based on the dose-response curves (Figure 103), irradiances eliciting maximum first positive curvature, 5 and 7 kiloergs cm⁻² for *Avena* and *Zea*, respectively, were used for phototropic stimulation.

After irradiation, groups of 500 coleoptile tips were placed in 15 ml distilled water and allowed to remain in the dark at 25° for 3 hours. The aqueous diffusates were frozen in liquid nitrogen and kept at -20° until enough diffusate had been collected for fractionation into the acid, basic, and neutral components, using procedures described in detail by Larsen.⁽³⁾ The neutral and basic components were discarded since they showed no auxin activity. In a number of experiments with the *Zea* coleoptiles, the tips were extracted with diethyl ether at 2 to 4° for ca. 16 hours. The extracts were similarly fractionated.⁽³⁾

The acid components were spotted on a chromato-

graphic strip and subjected to ascending isopropanol: ammonia (28%):water, 10:1:1, v/v/v. The resultant chromatogram was divided into 10 or 20 equal sections, each representing 0.1 or 0.05 Rf units. Each section was eluted with purified methanol and the evaporated eluates were assayed for auxin using either the *Avena* section test⁽⁴⁾ or the *Avena* curvature test.⁽³⁾

To evaluate the effect of light on the relationship between the auxins, it was necessary to describe quantitatively the auxin activities at different loci on the

FIG. 103.—Phototropic dose-response curves for unilateral irradiated coleoptiles. A, *Avena* (See Part II of this series of reports); B, *Avena* and C, *Zea* (present work). Irradiation time: 15 seconds for *Avena*, 22 seconds for *Zea*. Coleoptiles were exposed to red light, incident energy ca. 0.6 mW cm^{-2} , for one hour before phototropic stimulation.

chromatograms. Summation of the net increases in length of the test sections upon assay of the chromatogram segments about one locus is not valid, since response to known auxins in the section test is generally some logarithmic function of the concentration. Where the auxin is available in relatively pure form, viz., indoleacetic acid (IAA), concentration response curves can be constructed. Biological responses of individual chromatogram segments at the Rf of IAA may then be summed as IAA "equivalents." But for the uncharacterized auxins, whose concentration-response curves and intercepts (threshold) were not known, no such determination could be made. A method for the summation of the activities of chromatogram segments was, therefore, derived (see Appendix) and used in the present study. It should be stressed that qualitatively similar inferences may be drawn from comparison of the summed net growth increments of the test sections assaying the segments about one chromatogram locus.

For the curvature test, responses are an approximately linear function of auxin concentration within the proportionality range. Hence, for quantitative comparison, we have summed the individual average degrees of curvature obtained by assay of the different segments about one locus, since each datum was within the linear range.

PROGRESS REPORT

Equilateral Irradiation of Avena and Zea

Two auxins were repeatedly encountered upon chromatography of the diffusate from both unirradiated and irradiated excised coleoptile tips. Results of one experiment with *Avena* are shown in Figure 104. One auxin has the migration rate of IAA (Rf 0.3). The second has a Rf of ca. 0.65 and will be designated "P." Small amounts of a third auxin "F" traveling at the solvent front, are obtained from unirradiated tips (Figure 104A). The activity in this region of the chromatogram is generally less when the tips are exposed to light (Figure 104B). These histograms suggest that light reduces the activities at P and possibly at F loci and increases the activity at the Rf of IAA.

FIG. 104.—Bioassay of the chromatograms of the aqueous diffusate from *Avena* coleoptile tips, ether soluble acid fraction. A, unirradiated, 1350 tips; B, equilaterally irradiated, 1500 tips.

A more quantitative evaluation of the effect of light is given in Tables 44 and 45, which summarize several experiments. Irradiation causes a drop of P and F and an increase in IAA. The ratio of IAA to P and F is consistently increased. We suggest, hypothetically, that light induces the transformation of P and/or F to IAA.

Data from Table 45 (*Zea*) indicate that both IAA and P increased following treatment with red light. Since we have found, as has Briggs,⁽⁵⁾ that greater first positive curvature of the *Zea* coleoptile occurs after exposure to red light, all the subsequent experiments with the *Zea*

coleoptile incorporated one hour preexposure of the intact seedling to red light.

To investigate more effectively the action of light on F, P, and IAA, we increased the yields of these auxins by extracting the *Zea* tips at low temperature with ether (see Methods). Besides IAA, P, and F, auxin activity appeared at a new locus, Rf 0.4–0.45. This activity will be termed "M." A quantitative tabulation of the data is given in Table 46. Here again, light apparently causes an increase of IAA at the expense of the other auxins.

Unilateral Irradiation of *Zea*

Within one to two minutes after irradiation each tip was split into irradiated and shaded half, and each

TABLE 44

EFFECT OF EQUILATERAL IRRADIATION ON AUXINS
DIFFUSING FROM TIPS OF *Avena* COLEOPTILE

	IAA+ P+F (mm)	IAA (%)	P (%)	F (%)	IAA/ P+F
Unirradiated					
1350 tips.....	7.13	4	83	12	0.05
1400 tips.....	9.54	20	76	5	0.23
Irradiated					
400 tips.....	2.34	54	46	0	1.19
900 tips.....	6.81	32	67	2	0.46
1500 tips.....	4.89	46	54	0	0.86

TABLE 45

EFFECT OF GREEN AND RED WORK LIGHTS ON AUXINS
DIFFUSING FROM TIPS OF *Zea* COLEOPTILE EQUI-
LATERALLY IRRADIATED WITH BLUE LIGHT

	IAA+ P+F (mm)	IAA (%)	P (%)	F (%)	IAA/ P+F
Green					
Unirradiated					
750 tips.....	5.67	88	12	0	7.5
Irradiated					
750 tips.....	6.86	92	8	0	11.9
Unirradiated					
700 tips.....	7.16	71	29	0	2.5
Irradiated					
700 tips.....	2.77	91	9	0	9.7
Red					
Unirradiated					
750 tips.....	14.19	74	23	3	2.8
Irradiated					
750 tips.....	9.48	80	17	4	3.9
Unirradiated					
700 tips.....	8.95	90	10	0	9.5
Irradiated					
700 tips.....	7.19	98	2	0	16.8

group of half-tips extracted with ether. The acid fraction was then chromatographed and bioassayed. As with the whole tips, auxin activity was found at the F, P, M, and IAA loci; in the irradiated sides there was a decrease of activity at P and F and an increase at the IAA region. These relations are described quantitatively by data from experiments with sets of paired experiments summarized in Table 47. Here also the ratios of

TABLE 46

EFFECT OF EQUILATERAL IRRADIATION ON AUXINS
EXTRACTED FROM TIPS OF *Zea* COLEOPTILES

	IAA+ M+P+F (mm)	IAA (%)	M (%)	P (%)	F (%)	IAA/M +P+F
Unirradiated						
250 tips.....	5.02	12	5	66	17	0.13
Irradiated						
260 tips.....	4.57	30	16	49	5	0.42
Unirradiated						
350 tips.....	3.35	64	4	25	7	1.75
Irradiated						
350 tips.....	2.02	72	0	19	9	2.50
Unirradiated						
150 tips.....	4.10	35	9	51	5	0.53
Irradiated						
175 tips.....	3.64	38	23	39	0	0.63

TABLE 47

EFFECT OF UNILATERAL IRRADIATION ON AUXINS EX-
TRACTED FROM LIGHT AND DARK HALVES OF *Zea* CO-
LEOPTILE TIPS IMMEDIATELY AFTER IRRADIATION

	IAA+ M+P+F (mm)	IAA (%)	M (%)	P (%)	F (%)	IAA/M +P+F
600 tips						
Dark half...	2.93	42	3	43	12	0.72
Light half...	3.45	48	0	48	3	0.94
620 tips						
Dark half...	4.60	51	10	37	3	1.04
Light half...	3.15	84	0	15	1	5.30

IAA to the remaining auxins are increased in the tissue exposed to light.

Characteristics of P

Thermal Lability.—Earlier we suggested that light may induce the transformation of P and/or F to IAA. Consistent with this reference is the observation that heating of P and F *in vitro* converts them to IAA. An acid fraction from the aqueous diffusate from 1200 unirradiated *Avena* tips was chromatographed. The segments between Rf 0.15 and 0.45, which encompassed the IAA, were eluted and assayed. The auxin distribution in these segments is shown in Figure 105A, solid line. The remainder of the chromatogram between Rf 0.45 and 0.95 was eluted with ether. This eluate was re-

duced to dryness in a water bath at 97° within several minutes. It was again taken up in ether and spotted for chromatography. Auxin activities of the segments of the second chromatogram are shown in Figure 105B. Significant activity was found only at the Rf of IAA; little remained in the higher Rf's. Without the heat treatment (broken lines, Figure 105B) there was no significant conversion of the faster moving components, P and F, to IAA.

Basipetal Transport.—The acid fraction of the aqueous diffusate from 8000 to 9000 *Avena* tips (see Methods) was chromatographed (9 strips). The P areas between Rf 0.50 to 0.85 were eluted with methanol. The evaporated eluates were taken up in ether and distributed equally among 6 agar blocks (1.5%, 1 × 8 × 11 mm); (for method, see Reference 3). After the blocks were equilibrated for 2 hours, they were cut into three equal longitudinal strips and used as donor

FIG. 105.—A, Bioassay of chromatogram segments between Rf 0.15 and 0.45, of aqueous diffusate, acid fraction, of unirradiated *Avena* coleoptiles (1200 tips). Broken line indicates the distribution of activity on chromatogram segment, Rf above 0.45, in a comparable experiment with ca. the same number of tips. B, Solid lines: bioassay of above chromatogram (A) segments between Rf 0.45 and 0.95 that were eluted with ether, heated and rechromatographed. Broken lines: bioassay of chromatogram segments between Rf 0.45 and 0.95 where gaseous N₂ at 23° was used instead of heat to reduce the volume of the eluates before rechromatography.

TABLE 48

EXPERIMENTS ON BASIPETAL TRANSPORT OF P

		IAA (mm)	P (mm)	F (mm)
1	Donor block*		2.10	
	Tissue		0.58	
	Receiver block	0.16	0.08	0.06
2	Donor block*		2.75	
	Tissue	0.12	0.24	
	Receiver block			1.0

* Donor blocks in 1 and 2 contain P component diffused from 8000 and 9500 *Avena* tips, respectively.

blocks. Three subapical *Avena* coleoptile sections, 5 mm, cut 2 mm from the tip, were placed on an agar receiver block, 1 × 8 × 11 mm, maintaining normal vertical orientation. The coleoptile sections were held vertically through holes in a thin rigid plastic strip bridging the receiver block. A single donor was placed on the apical ends of each 3 coleoptile sections. Eighteen such assemblies were made with P component in the donor, matched by 18 control assemblies with blank chromatogram eluate in the donor. After 3 hours, the donor blocks, tissues, and receiver blocks were separated and immersed in ether for low temperature extraction.

FIG. 106.—*Avena* curvature bioassay of chromatogram of aqueous diffusate, acid fraction, of *Avena* coleoptiles (750 tips).

Table 48 is the summed activities at the various chromatogram loci of each of the extracts. These data show that P is not transported basipetally through subapical tissues of the *Avena* coleoptile.

The above inference is further substantiated by the inactivity of P in the *Avena* curvature test (compare Figure 106 to Figure 104A). There was a trace activity at the P locus. Virtually all of the activity was at the Rf corresponding to that of IAA.

Absorption Spectrum.—The acid fraction of aqueous diffusates from 6600 *Avena* tips was chromatographed. Chromatogram segments between Rf 0.5 and 0.8 were pooled and eluted with methanol. The evaporated eluate was dissolved in 5 ml twice-distilled water for determination of absorption spectra in the visible and ultraviolet regions. No absorption bands in the visible spectrum were found. In the uv, there was a minimum absorption band at 257 mμ, a maximum at 272 mμ, with a secondary absorption band at 287 mμ (Figure 107). The absorption spectrum shows little fine detail, and suggests the presence of underlying impurities.

DISCUSSION

We have shown that aqueous diffusates and ether extracts of *Avena* and *Zea* coleoptile tips, when chro-

matographically resolved, possess at least three auxins other than IAA. On the basis of section test bioassay, the major activities are at the Rf of IAA in the ammonia-isopropanol system, ca. 0.35, and a broader band, P, at Rf ca. 0.7. Some activity is found also at the solvent front (F) and between IAA and P (M).

P is an acidic auxin which is active in the *Avena* section test and not the curvature assay. It shows little, if any, basipolar movement, and is readily converted to IAA *in vitro* by heat. The uv absorption spectrum of a partially purified preparation of P possesses spectral characteristics of indolyl compounds. Is P identical with any of the characterized native auxins? In the ammoniacal chromatographic solvent systems used here, indole-3-ethylacetate has an average Rf of 0.75. It breaks down to some extent in this alkaline system to yield IAA, which appears at Rf 0.34. However, the ester may be ruled out since it, unlike P, does not behave as an organic acid in solvent partition and is not converted to IAA by the mild heat treatment. Indoleacetonitrile, whose Rf, 0.77, is similar to that of P, moves to some extent with organic acids on fractionation.^(6, 7, 3) However, it also is not converted to IAA and M by the heat

FIG. 107.—Ultraviolet absorption spectrum of the P component from 6000 *Avena* tips.

treatment and, unlike P, is active in the curvature test. Chromatograms of indoleacetaldehyde in the ammoniacal system showed no activity at any Rf, and the aldehyde remains in the neutral phase on solvent fractionation.

Indolepyruvic acid (IPyA) is unstable in the ammoniacal chromatography and to mild heat, breaking down into several substances with auxin activity, one of which is IAA.^(8, 9) The Rf of IPyA *per se* in the alkaline system cannot be defined (compare References 8 and 10).

Figure 108 shows results of bioassay by the section test and chromogenic spray of chromatograms of crystalline IPyA developed in the ammoniacal solvent. Three areas out of the six that developed color were active in the section test. The most active area falls at the IAA locus. Some activity remained at the origin, and low activity was found at the locus of P, Rf 0.6–0.9. The latter, area f (Figure 108), developed a color similar to that of IAA on treatment with FeCl₃ in dilute HClO₄. Indole-3-acetamide, which also gives a crimson

FIG. 108.—Bioassay (A) and color development (B) of chromatograms of indolepyruvic acid (IPyA) developed in isopropanol:ammonium hydroxide:water (10:1:1). Solid line histogram was developed from chromatogram spotted with 100 μg IPyA, broken lines from 10 μg IPyA. The spots along the chromatogram were developed by spraying the FeCl₃ in dilute HClO₄; the broken lines indicate areas fluorescent under uv light, before treatment with the color reagent.

color and is hydrolyzable to IAA, is apparently one of the decomposition products of IPyA upon alkaline chromatography.⁽¹¹⁾ Therefore, area f (Rf 0.6–0.9) was eluted with methanol and heated, rechromatographed, and the chromatogram assayed by the section test. All of the activity was found again at Rf 0.6–0.9. Thus, the breakdown product of IPyA, f, is apparently not P, which is converted to IAA by heat.

It is also unlikely that IAA complexes occurring in plant tissues, such as ascorbigen and indolylacetyl peptides, are identical with P. Ascorbigen does not partition as an acidic compound and is in itself inactive in the *Avena* section test. Furthermore, it breaks down in the ammoniacal chromatographic system to several biologically active compounds.⁽¹²⁾ The peptides, indolylacetyl-aspartate and -glutamate, are biologically active but are of dissimilar solubility and Rf to that of P. The slope of the concentration-response curve of indolylacetyl-glutamine parallels that of IAA (Reference 1, p. 616), unlike the slope for the curve of P (Figure 109).

Thus, on the basis of solubility and partition characteristics, thermal conversion to IAA, chromatographic behavior, and relative activity, P is apparently not any of the chemically characterized native auxins.

Because of the variation between experiments in the observed activity per tip, emphasis has been placed on ratios between the auxins within each experiment. However, irradiances in the first positive region consistently decreased the summed activities of the various auxins in the coleoptile tip. The average decrease of 7 separate experiments was $18 \pm 2.6\%$. This decrease may be attributed in large part to a drastic reduction in the amount of P. Immediately after irradiation of the tip, the ratio of IAA to the remaining auxins rises as much as

FIG. 109.—Concentration-response curves in the *Avena* section test. Solid line: IAA; Broken line: relative responses to serial dilutions of P.

TABLE 49

RATIO OF IAA TO M, P, AND F FOR EQUILATERALLY (e) IRRADIATED AND UNIRRADIATED *Avena* AND *Zea* TIPS AND FOR LIGHT AND DARK HALVES OF UNILATERALLY (u) IRRADIATED *Zea* TIPS

EXPERIMENT No.	IAA/M+P+F		LIGHT/DARK
	Light	Dark	
1 (e)	0.86	0.23	3.7
2 (e)	11.9	7.5	1.6
3 (e)	9.7	2.5	3.9
4 (e)	3.9	2.8	1.4
5 (e)	16.8	9.5	1.8
6 (e)	0.42	0.13	3.2
7 (e)	2.5	1.75	1.4
8 (e)	0.63	0.53	1.2
9 (u)	0.94	0.72	1.3
10 (u)	5.3	1.04	5.1
Mean			2.4

If 1 is the expected mean of light/dark, then $\chi^2 = 38.8$, a chance probability of <0.01 .

five-fold in *Avena* and two- to three-fold in *Zea*. This change in ratio is caused by both an increase in IAA and a decrease in P. In each of 10 experiments (Table 49), the ratio of IAA to the other auxins is higher in the light tip than in the dark tip, both for the equilateral and the unilateral irradiation. The quotients of these ratios are in all instances greater than unity; the probability that this would occur by chance is extremely small. In other words, IAA appears to increase at the expense of the other auxins as an immediate consequence of exposing the coleoptile tip to blue light.

Summarizing, we find that there are auxins other than the hormone IAA in the coleoptile tip, that these auxins may be associated with IAA biogenetically, and that relatively low irradiances in the blue spectral region change the ratios between these various auxins. The relations of these observations to the phototropic response will be discussed in the following report.

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REFERENCES

- Gordon S. A. and M. Eib. Auxin transport in the phototropic response. *Plant Physiol.* 31 (Suppl.), XIV (1956).
- Thimann, K. V. and G. M. Curry. Phototropism and phototaxis. *Comparative Biochemistry*, ed. M. Florkin and H. S. Mason. Academic Press, New York, 1960, pp. 243-309.
- Larsen, P. Growth substances in higher plants. *Modern Method of Plant Analysis*, ed. K. Paech and M. W. Tracy. Springer-Verlag, Berlin, 1955. Vol. 3, pp. 565-625.
- Bentley, J. A. Analysis of plant hormones. *Methods of Biochemical Analysis*, ed. David Glick. Interscience Publ., New York, 1962. Vol. 9, pp. 75-125.
- Briggs, W. R. Red light, auxin relationship and phototropic responses of corn and oats coleoptiles. *Am. J. Botan.* 50, 196-207 (1963).
- Bentley, J. A. The states of auxin in the plant. *Handbuch der Pflanzenphysiologie* 14, 609-619 (1961).
- Jones, E. R. H., H. B. Henbest, G. F. Smith and J. A. Bentley. 3-Indolylacetonitrile, a naturally occurring plant growth hormone. *Nature* 169, 485 (1952).
- Bentley, J. A., K. R. Farrer, S. Housley, G. F. Smith and W. C. Taylor. Some chemical and physiological properties of 3-indolepyruvic acid. *Biochem. J.* 64, 44-49 (1956).
- Bitancourt, A. A., A. P. Nogueira and K. Schwarz. Pathway of decomposition (catabolic lattice) of indole derivatives. *Plant Growth Regulation* (4th Intern. Conf. on Plant Growth Regulation). The Iowa State Univ. Press, Ames, Iowa, 1961, pp. 181-198.
- Stowe, B. B. and K. V. Thimann. The paper chromatography of indole compounds and some indole-containing auxins of plant tissues. *Arch. Biochem. Biophys.* 51, 499-516 (1954).
- Jepson, J. B. Indoles and related Ehrlich reactors. *Chromatographic Techniques*, ed. I. Smith. Heinemann Medical Books, Ltd., London, 1964, pp. 114-138.
- Kutacek, M. F., F. Icha, M. Valenta, J. Tupy and L. Holkova. Untersuchungen über Indolderivate in Rosenkohl. *Chem. Listy* 52, 531-536 (1958).

APPENDIX

We assume that the responses to the different auxins encountered are linear functions of the logarithm of their concentrations. Auxin response curves in the stoichiometric range (Figure 109) may be expressed as

$$r = b \log_{10} c + a \quad (1)$$

where r = response (net mm elongation), b = slope, c = concentration of the auxin, and a = intercept.

Then

$$\frac{r - a}{b} = \log_{10} c. \quad (2)$$

Exponentiating (2),

$$c = 10^{(r-a)/b} = \frac{10^{r/b}}{10^{a/b}}. \quad (3)$$

Let

$$k = 10^{-a/b}, \text{ a constant for each auxin;}$$

then

$$c = k 10^{r/b}. \quad (4)$$

The total concentration (C) of an auxin in one area of a chromatogram is the sum of the concentrations in the separate chromatogram segments ($\sum c_i$) within that area, i.e.,

$$C = \sum_{i=1}^n c_i.$$

Since, by (4)

$$c_i = k 10^{r_i/b},$$

$$C = k \sum_{i=1}^n 10^{r_i/b}. \quad (5)$$

Let

$$R = a + b \log C,$$

where R is the total response (mm) elicited by concentration C . Then

$$\begin{aligned} R &= a + b \log \left(k \sum_{i=1}^n 10^{r_i/b} \right) \\ &= a + b \left[\log k + \log \left(\sum_{i=1}^n 10^{r_i/b} \right) \right] \\ &= a + b \left(-\frac{a}{b} \right) + b \log \left(\sum_{i=1}^n 10^{r_i/b} \right) \quad (6) \\ &= b \log \left(\sum_{i=1}^n 10^{r_i/b} \right). \end{aligned}$$

We used the above procedure to sum the auxin activities of grouped segments of a chromatogram at one locus. The slope, b , of the concentration-response curve (within the proportionality range) for any auxin was obtained by determining the net mm elongation in the section test to serial dilutions of the auxin eluted from a single activity area. Figure 109, for example, shows the concentration-response curve for IAA and the relative concentration response of the auxin P obtained from coleoptile tissues. Unless otherwise indicated, all tabular data were based on the adjusted total response R (Equation 6), of the *Avena* test sections.

HORMONAL RELATIONS IN THE PHOTOTROPIC RESPONSE. IV. THE MOVEMENT OF ENDOGENOUS AND C^{14} -LABELED INDOLEACETIC ACID IN PHOTOTROPICALLY STIMULATED ZEA COLEOPTILES

Jane Shen Miller and Solon A. Gordon

PURPOSE AND METHODS

We have shown that aqueous diffusates and ether extracts of *Avena* and *Zea* coleoptile tips, when chromatographically resolved, possess at least three auxins, M, P, and F, in addition to indoleacetic acid (IAA). (See Part III of this series of reports.) Light in the blue spectral region (440 m μ) causes P and possibly F to decrease and IAA to increase in the coleoptile tips. A previous preliminary study in this Laboratory indicated that IAA is not laterally transported following phototropic stimulation. If lateral movement of IAA does not occur, and IAA is the hormonal auxin mediating the influence of the tip on the subjacent tissue, it

becomes necessary to postulate that phototropism involves two hormones. One hormone would move laterally in the phototropically sensitive tissues of the tip and cause a difference in the amount of IAA basipetally transported. We have, therefore, undertaken to reexamine the movement of IAA in the phototropically stimulated coleoptile.

Groups of 15 etiolated coleoptile tips (5 mm) of *Zea mays* (Burpee Snowcross), were mounted vertically on a stainless steel razor blade, 3 $\times$ 35 mm, so that the sharp edge penetrated ca. 1 mm into the base of the tip. The split basal ends of the coleoptile tips on each side of the razor blade rested on a 1.5% agar block (1 $\times$ 5 $\times$ 35

mm). The tips were oriented so that the plane of the blade was normal to and bisected the longer transverse axis of the coleoptile (the axis passing through the vascular bundles). This facilitated irradiation parallel to the long axis, which enhances the phototropic response.⁽¹⁾

For the study of the movement of exogenously applied IAA, C¹⁴-methylene labeled IAA (in 0.1% Tween-20) was applied to the coleoptile tips with a fine camel-hair brush. The specific activity of the IAA was 13.3 c/M. The tips were either irradiated with blue light, 1) immediately after C¹⁴-IAA application; 2) 15 min after application, or 3) not irradiated.

Accordingly, the experiment was repeated using a more specific test for IAA, the *Avena* curvature test, to assay the chromatogram segments. Activity appeared only at the Rf of IAA. Table 50 shows the effect of unilateral irradiation on the distribution of IAA in the tip-block assemblies 2.5 hours after irradiation. The blocks under the "dark" halves of the coleoptile tips had more IAA than those under the "light" halves. However, less IAA was obtained from the tissues of the dark sides of the irradiated tips. On the basis of the paired differences between the dark half-tips plus block and the light half-tips plus blocks, there is no significant lateral inequality between the two sides.

TABLE 50
IAA DISTRIBUTION IN LIGHT AND DARK HALVES OF *Zea* COLEOPTILE TIPS AND IN AGAR BLOCKS
SUBTENDING THE HALF-TIPS 2.5 HOURS AFTER UNILATERAL IRRADIATION*

NUMBER OF TIPS	AGAR BLOCK				COLEOPTILE TIPS			
	Total Degrees Curvature	Light Half (%)	Dark Half (%)	Δ D-L	Total Degrees Curvature	Light Half (%)	Dark Half (%)	Δ D-L
300.....	7	43 (1)	57 (1)	14	32	69 (2)	31 (1)	-38
350.....	19	47 (1)	53 (1)	6	24	67 (2)	33 (1)	-34
500.....	43	33 (3)	67 (3)	34	83	58 (2)	42 (3)	-16
2000.....	62	45 (2)	55 (3)	10	73	64 (3)	36 (3)	-28
Mean.....				16 ± 6.2				-29 ± 4.8
P.....				0.09				0.01

* Values in degrees are the summed mean curvatures obtained by assay of the individual chromatogram segments about the IAA locus. The number of the chromatogram segments summed is given in parentheses.

Mean paired differences: (Dark tip + block) - (Light tip + block) = -13 ± 10.5, P = 0.3.

Two and one-half hours after unilateral irradiation, the tip-block assemblies were disassembled. The tips were split into irradiated and shaded halves. The split tips and their subtending agar blocks were extracted separately with cold ether. The methods for extraction, fractionation, chromatography, and auxin bioassays were similar to those described in the preceding study (Part III).

For the C¹⁴-IAA experiments, the chromatogram of the extracts of the tip-block assemblies were divided into 10 equal segments, each representing 0.1 Rf unit. Each segment was eluted into a glass vial with methanol plus two ether washings. Fifteen ml of liquifluor (diluted with toluene and ethanol) were added to each evaporated eluate. The solution was counted directly on an automatic Tri-Carb liquid scintillation spectrometer.

PROGRESS REPORT

Endogenous Auxins

In a preliminary study using *Avena* section test for auxin bioassay, we found IAA but essentially no P or F in the extracts of the half-tips 2.5 hours after irradiation. IAA was also the predominant auxin in the agar blocks.

C¹⁴-Labeled IAA

The effect of unilateral irradiation of the coleoptile tips on the movement of exogenously supplied C¹⁴-labeled IAA was determined (see Methods). After disassembling the tip-block assemblies, the different components were extracted with ether and evaporated in the vials. The residual tips and blocks were dried in glass vials under an infrared lamp. Liquifluor was added to the evaporated eluates and the dried residues were counted on the scintillation counter. Results of the above transport experiments are given in Table 51.

The unirradiated control indicates that our technique was of good precision, 50% ± 0.6 of the total activity applied being found in the half-tip assemblies. There is no significant inequality in IAA distribution on the light or the dark side for both the tip and the block when irradiation was given 15 minutes after C¹⁴-IAA application. The effect of the light on the tissue may have been masked by the premovement of the applied auxin before exposure.

When the tips were irradiated immediately after C¹⁴-IAA application, the auxin distribution among the different components of the assemblies follows the same

trend as the endogenous IAA (compare Table 50 to 52). Table 52 shows the paired differences of C¹⁴-IAA distribution of the tips irradiated immediately upon IAA application. There is no inequality of total amounts of C¹⁴-IAA on the two sides. The light half tissues contained slightly more IAA than those on the dark side, though the difference was not significant. However, the difference between the blocks is significant; the dark blocks contained more IAA than the light.

The previous study (Part III of this series) has shown that immediately after equilateral irradiation, the ratio of IAA to the remaining three auxins (P and F and M) increases. With unilateral irradiation, the ratio is much higher in the light half tissues than it is in the dark. There are more of the other auxins and less IAA on the dark side than on the light side of the tip. Several hours after irradiation of the excised tip, all of the auxins other

than IAA have disappeared. This suggests that formation or supply of the other auxins in the tip is not maintained after excision, and from the heat conversion experiment, we may infer that P-F components are converted to IAA in the tip. The present observations suggest that this conversion is accelerated by light.

Tables 50 and 52 show that several hours after unilateral irradiation of a tip-block assembly, the amount of IAA in the tip plus block on the light side is not significantly different from that in the side away from the light. However, the blocks of the shaded side have more of the auxin. Several explanations of this relationship may be considered. First, a rise in IAA on the light side immediately after irradiation, derivable from an accelerated conversion of P to IAA, might impair the rate of downward transport of IAA. But the capacity, and to some extent the velocity, of basipetal transport of IAA

TABLE 51
PER CENT DISTRIBUTION OF C¹⁴-IAA IN LIGHT (L) AND DARK (D) HALVES OF UNILATERALLY IRRADIATED *Zea* COLEOPTILES. I, UNIRRADIATED; II, IRRADIATED 15 MINUTES AFTER C¹⁴-IAA APPLICATION TO TIPS*

	I (HALF-TIP)	II		
		($\bar{D}$)	($\bar{L}$)	($\bar{D} + S\bar{D}$)†
<i>Tip</i>				
Ether extract (IAA).....	19.3±0.6	19.4±1.1	21.1±2.0	-1.65±1.8
Residual activity.....	4.3±0.2	3.0±0.6	3.1±0.4	-0.42±0.65
Total.....	23.6±0.8	22.4±1.4	24.1±1.7	-1.75±1.96
<i>Receiver block</i>				
Ether extract (IAA).....	23.5±0.7	24.4±1.4	24.0±1.5	-0.37±1.77
Residual activity.....	2.9±0.2	2.3±0.3	2.8±0.2	-0.52±0.2
Total.....	26.6±0.7	26.7±1.2	26.6±1.7	-0.03±1.8
<i>Total (tip and block)</i>	50.0±0.6	49.0±2.1	51.0±2.1	-1.9±3.06

* Based on the means of I, three experiments and II, six experiments (30 tips per experiment). The following amounts of C¹⁴-IAA were applied per tip in the replicate experiments: I, 4.8, 5.7, and 6.0 mμg; II, 3.7, 3.9, 3.6, 5.1, 5.1, and 8.2 mμg.
† $\Delta = (D) - (L)$; $\bar{\Delta}$ = mean of (D) - (L).

TABLE 52
PAIRED DIFFERENCES OF C¹⁴-IAA PERCENTAGE DISTRIBUTION BETWEEN LIGHT AND DARK HALVES OF UNILATERALLY IRRADIATED *Zea* COLEOPTILES. IRRADIATION FOLLOWED IMMEDIATELY THE APPLICATION OF C¹⁴-IAA TO TIPS

	Mean*	t	pt
<i>Tip</i>			
Ether extract (IAA).....	-2.0±1.4	1.47	0.2
Residual activity.....	-0.1±0.2	0.26	0.8
Total.....	-2.1±1.6	1.35	0.3
<i>Receiver block</i>			
Ether extract (IAA).....	+5.3±2.0	2.66	0.05
Residual activity.....	+0.8±0.2	3.90	0.01
Total.....	+6.3±2.0	3.12	0.02
<i>Total (tip and block)</i>	4.1±2.5	1.64	0.2

* Dark half minus light half. Based on the means of six experiments, 30 tips per experiment. The following amounts of C¹⁴-IAA were applied per tip in the replicate experiments: 3.3, 3.3, 3.8, 4.2, 5.5, and 8.0 mμg.

† Probability of the null hypothesis using paired differences.

increases with IAA concentration.⁽²⁾ Thus, one would expect a greater amount of auxin in the agar block on the light side. The converse is true.

Second, if we rule out from this work the lateral transport of IAA, there may be a specific transport form of IAA that is derived from P or P and F. There is less of the transport form in the tissue proximal to the light because of the competitive conversion of P or P + F to IAA.

Third, there may be a decrease in the rate of IAA basipetal movement in the tissues proximal to the light. Presumably arguing against this possibility are the observations that light *per se* does not decrease the rate of basipetal transport of auxin.⁽³⁻⁵⁾

The preceding explanations are speculative. We have substantiated that unilateral irradiation does cause more IAA to be delivered by the shaded side of the

coleoptile tip than by the irradiated side. However, the tissues on the irradiated sides have more IAA. Significantly, the total amounts of IAA are the same on the two sides of a tip plus block assembly after unilateral irradiation, when we evaluate the distribution of either endogenous IAA (Table 50) or exogenously supplied C^{14} -labeled IAA (Table 52). These observations do not support the hypothesis that a lateral movement of IAA occurs after phototropic stimulation. However, they do not preclude the lateral movement of a substance, essential for both the development of inequalities of auxin concentration and for phototropism, upon unilateral irradiation. Many experiments suggest that a transverse movement of a material stimulus does occur.^(1, 6, 7) We infer, therefore, that unilateral irradiation affects the relative amounts of IAA translocated from the light and dark sides of a tip into agar blocks or subapical tissues, without antecedent lateral transport

of this auxin. It would be desirable to reexamine the influence of light (equilateral) on the basipetal movement of endogenous auxins.

REFERENCES

1. Brauner, L. Über die Funktion der Spitzenzone beim Phototropismus der *Avena*-Koleoptile. *Z. Botan.* **43**, 467-498 (1955).
2. Naqvi, S. M. Transport studies of C^{14} -indoleacetic acid and C^{14} -2,4-dichlorophenoxyacetic acid in *Coleus* stem. Ph.D. Thesis, Princeton University, Princeton, N.J., 1963.
3. Overbeck, J. Van. Wuchsstoff, Lichtwachstumsreaktion bei *Raphanus*. *Rec. trav. botan. Néerl.* **30**, 537-626 (1933).
4. Reiff, B. and H. Van Guttentburg. Über der Förderung des polaren Wuchsstofftransports durch ATP. *Naturwissenschaften* **46**, 235-236 (1959).
5. Stewart, W. S. and F. W. Went. Light stability of auxin in *Avena* coleoptiles. *Botan. Gaz.* **101**, 706-714 (1940).
6. Briggs, W. R. Mediation of phototropic responses of corn coleoptiles by lateral transport of auxin. *Plant Physiol.* **38**, 237-247 (1963).
7. Pál, A. Über phototropische Reizleitung. *Jahrb. wiss. Botan.* **58**, 406-458 (1919).

AUXIN TRANSPORT IN CORN COLEOPTILE SECTIONS. I. THE EFFECT OF CARBOHYDRATE SUPPLEMENT ON TRANSPORT POLARITY, VELOCITY, AND CAPACITY

Shamshad M. Naqvi and Solon A. Gordon

PURPOSE AND METHODS

The kinetics of basipetal transport of the growth hormone indoleacetic acid (IAA) in most plant tissues are only explainable as an active transport phenomenon. The auxin is transported with velocities and flows much greater than those of simple diffusion, and with metabolic dependence. Since coleoptile sections are frequently used for the determination of transport parameters, sections that are etiolated and divorced from the endosperm, we considered that the transport characteristics might be modified by the limited availability of carbohydrate. The present study concerns itself with the effect of supplementary sucrose on the transport polarity, velocity, and capacity of C^{14} -labeled IAA in coleoptile sections. The labeled auxin was supplied to the tissues from donor blocks of agar; transported radioactivity was identified as C^{14} -IAA by chromatographic analysis.

Seeds of *Zea mays* L. (hybrid 64a X 22R) were soaked for two hours in warm tap water (60° C); the soaked seeds were planted on moist paper pads in plastic boxes and kept in the dark at 25° C. The seedlings were exposed to red light on the second day after soaking to inhibit mesocotyl elongation.

Donor blocks of 1.5% agar (11 X 8 X 1 mm) contained 0.4 mg/l of C^{14} -IAA purified by chromatography before use. Sucrose, when used, was incorporated into

the donor blocks at a concentration of 1%. For supplying different amounts of C^{14} -IAA at the same concentration, the thickness of the donor block was doubled (11 X 8 X 2 mm) or tripled (11 X 8 X 3 mm). These rectangular blocks were then divided into 12 equal blocklets. The receiver blocks were of similar agar concentration and size (11 X 8 X 1) but contained neither auxin nor sugar and were divided into approximately six equal blocklets.

Transport experiments were conducted with 7.0-mm sections taken from 1-2 mm below the tip of 96-hour-old etiolated coleoptiles. One donor blocklet was mounted on the physiological apex, and the base was placed on a receiver blocklet. For acropetal transport, the positions of donor and receiver were reversed without changing the normal orientation of the coleoptile section. Thus each assembly consisted of one coleoptile section with its donor and receiver blocklets. Six such assemblies were pooled together at the end of each time period, and the donors, tissues, and receivers were transferred to separately labeled vials for the determination of C^{14} activity. Each vial contained 15.0 of a toluene:Liquifluor: solvent⁽¹⁾ with predetermined backgrounds. β -emission of the vials was determined in a Packard Tri-Carb liquid scintillation counter.

In a separate 3-hour transport experiment, the receiver blocks were eluted with ethanol, and the eluate

was chromatographed with 5 μ g of unlabeled IAA (technique of Gordon and Paleg, Reference 2). Such an analysis showed that 90–93% of the radioactivity in the receivers could be recovered from the Rf of IAA, 0.37. The remainder of the activity was evenly distributed along the chromatogram.

All data presented under "Results" are the averaged means of three separate experiments. Student's "t" test was used for evaluation of statistical significance.

PROGRESS REPORT

Linearity of Auxin Transport

Donor blocks containing 0.4 mg/l C^{14} -IAA were applied to the coleoptile sections and the receiver blocks were assayed at half-hour intervals. Figure 110 shows that there is an almost linear increase in transported radioactivity up to 2 hours. Between 2 to 3 hours the rate of transport drops. Since the activity in the donor was reduced by about $\frac{1}{3}$ in 2 hours, leaving an auxin concentration that would yield a linear transport for 2 hours if it were applied initially, it is improbable that the change in rate in Figure 110 is caused by a limitation of donor C^{14} -IAA. Therefore, the possibility of carbohydrate limitation was investigated.

FIG. 110.—Basipetal auxin transport in etiolated corn coleoptile sections. Donor blocks without sucrose.

Effect of Sugar on C^{14} -IAA Transport

In these experiments the donor blocks contained 1% sucrose together with 0.4 mg/l of C^{14} -IAA; transport determinations were made for 5 hours. The results, presented in Figure 111, show that when donor blocks of $11 \times 8 \times 1$ mm were used, again a departure from linearity occurs at 2 hours. But in this instance the donor blocks had lost almost all their activity by 2 hours. By increasing the size of the donor blocks, keeping the concentration constant, C^{14} -IAA was supplied.

As shown in Figure 111, this resulted in the maintenance of linearity up to 4 hours.

What is the effect of sugar on the transport capacity and velocity of the etiolated sections? This may be determined from the data shown in Figure 112 comparing transport with and without sugar. Straight lines were fitted to the data by the method of least squares. Sugar does not significantly affect the velocity of C^{14} -IAA transport, but it does increase the transport capacity about 1.7-fold.

FIG. 111.—Effect of donor block size on the basipetal auxin transport of coleoptile sections. Donor blocks contained 1% sucrose.

When the radioactivity from donors, tissues, and receivers were plotted against time (Figure 113), it is clear that, after one hour, the basipetal C^{14} -IAA transport occurred against a concentration gradient. The possibility that the acropetal transport may have a higher sugar requirement than basipetal transport is not here indicated; there was no detectable radioactivity in the acropetal receivers even after a 5-hour transport period. Though the tissues did contain an appreciable amount of C^{14} activity, they still contained less than those tissues from the basipetal transport assemblies.

Concentration Effect on C^{14} -IAA Transport

The transport capacity and velocity was determined for donor blocks with 2 concentrations of C^{14} -IAA, 0.4 mg/l (A) and 0.2 mg/l (B), Figure 114. Transport rates were determined by least square minimization of the initial linear phase. Both transport velocity and ca-

FIG. 112.—Effect of sucrose on the velocity and transport capacity of auxin in coleoptile sections. A, with sugar; B, without sugar.

FIG. 113.—Polar transport of auxin against a concentration gradient. Broken line represents acropetal transport.

capacity were significantly greater with the higher concentration of auxin.

CONCLUSIONS

We may conclude, therefore, that when auxin is supplied in nonlimiting concentrations to corn coleoptile segments, basipetal transport occurs at first at a constant rate. This rate is limited by carbohydrate availability since subsequent rate decrease is reversed completely by exogenously supplied sugar. Such limitation appears consistent with an endergonic requirement for active transport. Indeed, even initial transport rate is increased by sucrose (compare Figure 112, A, with 112, B) though the longitudinal velocity of C^{14} -IAA movement

FIG. 114.—Effect of auxin concentration on the velocity and transport capacity of coleoptile sections. A, 0.4 mg/l; B, 0.2 mg/l.

in the present work, 20 mm per hr, is unaffected. It is pertinent that Goldsmith and Thimann⁽³⁾ were able to extend the linear period of auxin transport in *Avena* sections when the organ segments were increased from 7 to 20 mm in length. They proposed that the rate change was caused by a limitation of auxin availability through diversion into a static pool of auxin unavailable for transport. We suggest that the rate retention may be caused in part by the greater reserve of the longer segment. Using 5-mm *Avena* coleoptile sections, Gordon and Eib (unpublished) were also able to alter the period of constant transport rate from 3 to 5 hours by sucrose supplement.

The observation of van der Weij⁽⁴⁾ that transport occurs against a concentration gradient is further substantiated by Figure 113. The transport was strictly basipetal; no acropetal transport was observed even when the donors contained sucrose.

It is of interest that a concentration of auxin that

does not limit transport in the absence of sugar (Figure 110) does so in the presence of sugar (Figure 111). Since the transport velocity is not significantly affected by sucrose (Figure 112), we suggest that the carbohydrate causes a more rapid outflow from the donor block by increasing the concentration gradient at the interface. Either the sugar-enhanced transport capacity, or possibly a sugar-enhanced auxin catabolism, would contribute to such a gradient change.

Van der Weij⁽⁴⁾ stated that the velocity of auxin transport was independent of the concentration of the auxin. This conclusion is not supported statistically by his experimental data based on bioassay, which have a large variance and range. With the more precise measurements enabled by the C¹⁴-labeled auxin, we find (Figure 114) a velocity of 17.6 mm per hr at 0.2 mg/l and 20.0 mm per hr at 0.4 mg/l. The difference is significant at the 1 to 5% probability level. An analogous

effect of concentration on transport velocity has been observed with *Coleus* stems by Naqvi⁽¹⁾ and with bean petiole sections by McCready and Jacobs.⁽⁶⁾

REFERENCES

1. Naqvi, S. M. Transport studies with C¹⁴-indoleacetic acid and C¹⁴-2, 4-dichlorophenoxyacetic acid in *Coleus* stems. Ph.D. Thesis, Princeton University, Princeton, N.J., 1963.
2. Gordon, S. A. and L. G. Paleg. Observations on the quantitative determination of indoleacetic acid. *Physiol. Plantarum* **10**, 39-47 (1957).
3. Goldsmith, M. H. and K. V. Thimann. Some characteristics of movement of indoleacetic acid in coleoptiles of *Avena*. I. Uptake, destruction, immobilization and distribution of IAA during basipetal transport. *Plant Physiol.* **37**, 492-505 (1962).
4. van der Weij, H. G. Der Mechanismus des Wuchsstofftransports. *Rec. trav. botan. Néerl.* **29**, 379-496 (1932).
5. McCready, C. C. and W. P. Jacobs. Movement of growth regulators in plants. II. Polar transport of radioactivity from indoleacetic acid-(¹⁴C) and 2,4-dichlorophenoxyacetic acid-(¹⁴C) in petioles of *Phaseolus vulgaris*. *New Phytol.* **62**, 19-34 (1963).

AUXIN TRANSPORT IN ZEA MAYS L. COLEOPTILE SECTIONS. II. INFLUENCE OF GRAVITY ON THE TRANSPORT OF INDOLEACETIC ACID-2-C¹⁴

Shamshad M. Naqvi and Solon A. Gordon

PURPOSE AND METHODS

Geotropic curvature is a result of an unequal rate of growth of the upper and lower halves of a stimulated organ. These growth inequalities are generally considered to be caused by a lateral transport of the hormone auxin, elicited by the geotropic stimulus.⁽¹⁻³⁾ The validity of this explanation has recently been challenged by a number of workers.⁽⁴⁻⁶⁾ An alternate hypothesis proposes that reorientation⁽⁷⁾ and unilateral irradiation (this report) alter the rate of longitudinal auxin transport to and through reactive tissues rather than cause a lateral relocation of the hormone. Thus a difference in the rate of hormone transport proximal and distal to the stimulus direction would account for both the auxin and growth inequalities requisite for tropism. The preceding hypothesis is examined experimentally in this report, the first of a series, in which C¹⁴-labeled indoleacetic acid (IAA) is used to investigate the effect of stimuli, in the present instance, gravity, on the kinetics of auxin transport.

C¹⁴-IAA was purified before use by paper chromatography. Seven- or 10-mm coleoptile cylinders were cut 2 to 3 mm from the tips of 96-hour-old etiolated *Zea mays* L. seedlings. A donor block, containing 0.4 mg/l of C¹⁴-IAA, was placed on the morphologically apical end of each tissue. The donor block and coleoptile cylinder were placed on a receiver block for normal axial orientation. The whole assembly was inverted to shift

the direction of the gravitational field 180°. For a horizontally oriented coleoptile cylinder, a stainless steel razor blade (3 × 40 mm) was used to divide the receiver blocks into two equal halves; the blade passed through the agar into the base of the tissue cylinder, bisecting the tissue into two approximately equal halves (the vascular bundles were perpendicular to the razor blade).

Receiver blocks were counted with a Packard Tri-Carb scintillation counter using a solvent system of toluene: ethanol:Liquifluor.⁽⁸⁾ In another series of parallel experiments the receiver blocks were extracted with ethanol and the extract was chromatographed. Over 90% of the C¹⁴ activity could be accounted for as C¹⁴-IAA. No other band of activity appeared. The C¹⁴ activity in the receiver blocks was, therefore, considered to be solely C¹⁴-IAA. All these experiments were conducted at 25° in a dark-room. Manipulations were performed under phototropically inactive green illumination. Differences between means were evaluated by the "t" test.

PROGRESS REPORT

The data of Figure 115 indicate that inversion has no significant effect on either transport velocity (x-axis intercept) or transport capacity (slope). The velocity for 7-mm sections was 11-12 mm/hr and 12-13 mm/hr for 10-mm sections. These velocities agree well with those found by Gordon and Eib⁽⁹⁾ in comparable experiments with sections of *Avena* in normal orientation.

Coleoptile cylinders were then kept in normal and inverted orientation for 5 hours, with 2% sucrose and unlabeled auxin (0.4 mg/l) supplied in maintenance blocks, before transport determinations were begun. Under these circumstances (Figure 116), the abscissal intercept for normally oriented sections yields a calculated velocity of 12.10 ± 0.69 mm/hr, whereas in the inverted sections the velocity decreased to 9.80 ± 0.13 mm/hr. The difference is significant at the 2% level. Similarly, inversion decreased significantly (1% level)

FIG. 115.—Effect of inversion on C^{14} -IAA transport in *Zea mays*, var. Snowcross, coleoptile sections; IAA, 0.4 mg/l. Transport began immediately following inversion.

FIG. 116.—Inhibition of C^{14} -IAA transport in *Zea mays*, var. Snowcross, after 5 hours of inversion.

the slopes of the curves, i.e., the transport capacity, from 406 ± 11.4 cpm/hr to 333 ± 8.3 cpm/hr.

Another variety of corn (hybrid 64a $\times$ 22R) was then tested. The results with this variety, given in Figure 117, show an immediate effect of inversion on the C^{14} -IAA (0.2 mg/l) transport. Here the normally oriented sections transported 163 ± 1 cpm/hr with a velocity of 17.7 ± 0.2 mm/hr, while the inverted sections transported 113 ± 3 cpm/hr with a velocity of 15.7 ± 0.1 mm/hr. Chance probabilities for these differences are on the order of 0.1%.

It is of interest that the inverted sections which had been preexposed to red light (G.E. Ruby Red bulb) before the transport run, show a transport capacity of

FIG. 117.—Inhibition of C^{14} -IAA transport in *Zea mays*, hybrid 64a $\times$ 22R, coleoptile sections. IAA, 0.2 mg/l.

112 ± 4 cpm/hr, which is similar to that of sections not preexposed. However, the velocity is further decreased by the red light to 13.8 ± 0.3 mm/hr, a decrease statistically significant from both the normal and inverted tissues not exposed to red light. Since preexposure to red light increases the tropic sensitivity of tissues,⁽¹⁰⁾ probably through the mediation of phytochrome, we suggest that both the tropic enhancement and the impairment of hormone transport velocity have a common photoreceptor.

There are discrepancies in the literature with regard to the effect of orientation on auxin transport. Using bioassay methods, van der Weij⁽¹¹⁾ observed a slight inhibition of the amount of auxin transported to a block from inverted tissues. The single time used and the lack of variance data do not permit any rigorous inference from

his experiments about the effect of orientation on auxin transport. From similar experiments, Pfaeltzer⁽¹²⁾ concluded that the longitudinal force of gravity does not affect auxin transport. Recently, Hertel and Leopold⁽¹³⁾ reported an inhibition of C^{14} -IAA transport caused by an inversion of the coleoptile cylinders. However, one cannot determine from their data whether the inhibition was caused by an effect on transport velocity or on capacity. From the present study, we may conclude that the 180° inversion of tissues with respect to earth gravity impairs both the velocity and capacity of auxin transport. The immediacy of the change in auxin transport to an altered direction of gravity appears to de-

capacity of the lower tissues to translocate the hormone basipetally, and a decrease in transport capacity in the upper tissues, compared to the rates in vertically-oriented control tissues.

When the cylinders were completely bisected by a thin glass plate before being horizontally oriented, no difference in transport velocity developed (Figure 119). But again, the horizontal position elicits a change in the transport capacities. In the vertical halves the transport capacity averages 66 ± 1 cpm/hr, horizontal upper halves 60 ± 1 cpm/hr, and lower horizontal halves 73 ± 1.5 cpm/hr, differences are significant at the 2 to 5% level. Here, also, the transport capacity of lower

FIG. 118.—Auxin transport in partially split vertically and horizontally oriented *Zea mays*, hybrid 64a $\times$ 22R, coleoptile sections

pend on the plant species or variety, a dependence perhaps associated with the differences in the minimum presentation time among various plants.⁽¹⁴⁾

Reorientation of the coleoptile sections of corn (var. 64a $\times$ 22R) from the normal vertical position to the horizontal resulted in a 14% inhibition of transport capacity (Figure 118A); there was no significant change in the velocity of auxin transport. In these experiments transport in each tissue half above the partially inserted razor blade was measured. Since right and left halves of vertically oriented sections showed an identical transport rate, the average of these two halves was compared to the transport capacity and velocity in upper and lower halves of horizontally oriented sections (Figure 118B). The abscissal intercepts of vertical, upper, and lower halves all yield a calculated velocity of 18 mm/hr. Thus there was no effect of reorientation on transport velocity. However, the slopes show a transport capacity in the halves of 131 ± 1 cpm/hr (vertical), 51 ± 1 cpm/hr (upper), and 174 ± 2 cpm/hr (lower). These highly significant differences indicate that horizontal reorientation results in an increase in the

FIG. 119.—Auxin transport in totally split vertical and horizontal halves of *Zea mays*, hybrid 64a $\times$ 22R, coleoptile sections.

horizontal halves is higher than that of the vertical control, which, in turn, is higher than that of the upper horizontal halves.

It is surprising that most of the works interpreted in support of the lateral transport theory for geotropic response do not consider normally oriented controls with split bases.^(1, 2, 10, 15, 16) However, Gillespie and Briggs⁽¹⁵⁾ reported that when the endogenous auxin from 3 coleoptile tips was diffused into an agar block, the block produced a curvature of 30.6 ± 2.0 degrees in the *Avena* test. When 6 such sections (base split) were placed horizontally, they obtained 34.0 ± 1.6 degrees curvature from the lower half and 17.7 ± 1.2 degrees from the upper. Since 6 half coleoptiles may be expected to produce the same amount of auxin as 3 intact coleoptiles, the effect of horizontal placement on the auxin balance may be evaluated. If a lateral movement of auxin had occurred, the loss from the upper half should have become the gain in the lower. This does not appear to have occurred. The loss of 12.9 ± 2.5 degrees in the upper block is concomitant with a gain of 3.4 ± 2.6 degrees in the lower. Considering the two vertical halves as donating 50% of the total auxin diffused into the blocks, the horizontal lower half yielded 56.7% and the upper half 29.5%. Adding these we find a reduction of 14% induced by horizontal placement. This is in rather close agreement with our results (Figure 118A), where there occurs an inhibition of 14% in the auxin transport when the tissues are reoriented horizontally.

Similarly, using carboxyl-labeled IAA and *Avena* coleoptile sections, Gillespie and Thimann⁽¹⁶⁾ observed that in vertical halves 27 ± 2 cpm was recovered from the receivers after 165 minutes of transport. When the assemblies were placed horizontally the recovery from upper receivers was 25 ± 1 cpm and from lower 37 ± 1 cpm. The gain in the lower side cannot be explained by the loss on the upper.

We suggest that the lack of balance accountancy in the above-mentioned works cannot be explained simply by a lateral transport of the hormone upon geotropic challenge. On the basis of the present study, we propose that geotropic stimulation by horizontal placement of vertical organs causes a reduction of auxin transport capacity in the upper tissues, and an increase in that capacity in the tissues of the lower side. Since the growth rate of horizontally placed coleoptiles is virtually equal to the rate of the vertical organ,^(2, 17) the lower half must grow at a faster rate than the analogous tissue in the vertical organ, and the upper half must elongate at a slower rate than its vertical counterpart to produce a geonegative curvature. Indeed, series of marks along the upper and lower surfaces show that geostimulation

reduces the expansion rate of the upper and increases the expansion rate of the lower tissues.⁽²⁾ The difference in transport of auxin between the upper and lower tissues here observed would be entirely compatible with these relative expansions of tissues whose rates of elongation are auxin limited.

We cannot rigorously exclude the possibility that reorientation results in a simultaneously occurring lateral movement of C¹⁴-IAA together with the effect on transport capacity. However, in the totally split sections, where lateral translocation was excluded, we still observe a reduction in transport capacity in the upper halves and an enhancement in the lower.

REFERENCES

1. Dijkman, J. J. A quantitative analysis of the geotropical curvature in dicotyledons. *Proc. kon. Akad. Wet.* Amsterdam **36**, 748-758 (1933).
2. Dolk, H. E. Geotropism and the growth substance (English translation by F. Dolk-Hoek and K. V. Thimann). *Rec. trav. botan. Néerl.* **33**, 505-585 (1936).
3. Went, F. W. and K. V. Thimann. *Phytohormones*. Macmillan Press, New York, 1937.
4. Bünning, E., H. J. Reisener, F. Weygand, H. Simon and J. F. Klebe. Versuche mit radioaktiver Indolyllessigsäure zur Prüfung der sogenannten Ablenkung des Wuchshormonstromes durch Licht. *Z. Naturforsch.* **11b**, 363-364 (1956).
5. Ching, T. M. and S. C. Fang. The redistribution of radioactivity in geotropically stimulated plants pretreated with radioactive indoleacetic acid. *Physiol. Plantarum* **11**, 722-727 (1958).
6. Reisener, H. J. and H. Simon. Weitere Geotropismus Versuche mit radioaktiver β -Indolyllessigsäure (IES-2-C¹⁴). *Z. Botan.* **48**, 66-70 (1960).
7. Anker, L. On a geo-growth reaction of the *Avena* coleoptiles. *Acta Botan. Néerl.* **9**, 411-415 (1960).
8. Naqvi, S. M. Transport studies with C¹⁴-indoleacetic acid and C¹⁴-2,4-dichlorophenoxyacetic acid in *Coleus* stems. Ph.D. Thesis, Princeton University, Princeton, N.J., 1963.
9. Gordon, S. A. and M. Eib. Hormonal relations in the phototropic response: Polarity and velocity of auxin transport. Quarterly Report of the Biological and Medical Research Division, Argonne National Laboratory. ANL-5597 (1956), pp. 51-56.
10. Gillespie, B. and K. V. Thimann. Transport and distribution of auxin during tropistic response. I. The lateral migration of auxin in geotropism. *Plant Physiol.* **38**, 214-225 (1963).
11. Van der Weij, H. G. Der Mechanismus des Wuchsstofftransports. *Rec. trav. botan. Néerl.* **29**, 379-496 (1932).
12. Pfaeltzer, J. W. Lenjtekraft, groeistof en groei bij het coleoptil von *Avena sativa*. Dissertation, University of Utrecht, Netherlands, 1934.
13. Hertel, R. and A. C. Leopold. Versuche zur analyse des auxintransports in der koleoptile von *Zea mays* L. *Planta* **59**, 535-562 (1963).
14. Audus, L. J. The mechanism of the perception of gravity by plants. *Symp. Soc. Exptl. Biology* **16**, 196-226 (1962).
15. Gillespie, B. and W. R. Briggs. Mediation of geotropic response by lateral transport of auxin. *Plant Physiol.* **36**, 364-368 (1961).
16. Gillespie, B. and K. V. Thimann. The lateral transport of indoleacetic acid-C¹⁴ in geotropism. *Experientia* **17**, 126-129 (1961).
17. Navez, A. E. and T. W. Robinson. Geotropic curvature of *Avena* coleoptiles. *J. Gen. Physiol.* **16**, 133-145 (1933).

AUXIN TRANSPORT IN FLOWERING AND VEGETATIVE SHOOTS OF *COLEUS BLUMEI* BENTH.

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PURPOSE AND METHODS

There is considerable evidence that longitudinal transport of auxin in young monocot shoots is strictly basipetal.⁽¹⁻³⁾ Acropetal transport is essentially negligible except where auxin is introduced into the transpiration stream or where supraphysiological concentrations of auxin are employed.⁽⁴⁾ However, for the dicots there is no consistency in the reported findings on transport polarity. Nonpolarity⁽⁵⁻⁹⁾ as well as strict basipolarity^(8, 10, 11) has been reported, even for morphologically similar tissues. In view of this lack of agreement, the present work was undertaken. It deals with the polarity of transport of C¹⁴-labeled indoleacetic acid in stem sections of young vegetative and flowering shoots of *Coleus blumei*.

2-C¹⁴-methylene-IAA, specific activity 13.3 Ci/mole was stored in acetonitrile⁽¹²⁾ at a concentration of 10 μ Ci/ml. Before use in transport experiments, the IAA-C¹⁴ was purified by chromatography;⁽¹³⁾ the auxin was eluted from the chromatogram with methanol. An aliquot of the eluate was dried at room temperature under a stream of nitrogen, and the residue was taken up in 1.5% agar. From this agar donor blocks were poured, 5.5 $\times$ 8 $\times$ 1 mm in dimension, containing approximately 0.4 mg IAA-C¹⁴/l. This concentration is well below those that saturate the transport system, i.e., 1 to 5 mg/l.^(7, 14, 15) Receiver blocks were similar in dimension and agar composition but contained no auxin.

A single clone of *C. blumei* Benth. (var. Golden bedder) was used as a source of stem sections. The plants were grown from stem cuttings under an 18-hour day at 22° alternating with a 6-hour night at 18°. Illumination came from a mixture of 4500° K fluorescent tubes and tungsten lamps in a wattage ratio of 5 to 1. This supplied an irradiance of 2500 fc at plant level. The test sections were cut from the uppermost portion of the youngest internode (about 2 to 3 cm below the apex) from which a 5.0-mm segment could be readily taken. Two parallel razor blades were used as the cutting tool. Each section was ringed with vaseline to minimize movement of the IAA-C¹⁴ on the surface of the tissue under conditions of high humidity. For basipetal transport, the base of the stem section was placed on a receiver block; the donor block was then mounted on the top cut surface. For acropetal transport, the positions of the donor and receiver blocks were reversed. These transport assemblies were placed in humidified cham-

bers and kept in room light (fluorescent tubes, about 25 fc) at 22°.

After 3 hours, the donor blocks were removed and placed in vials that contained 15.0 ml of solvent (toluene:Liquifluor: ethanol) for scintillation counting; these were stored overnight at 2°.⁽¹⁴⁾ The tissues were sliced longitudinally into 3 pieces, which were placed directly into 15.0 ml of the scintillation solvent and stored at 2° for at least 24 hours. β -emission of the C¹⁴ from the samples was determined with a Packard Tri-Carb liquid scintillation counter.

The receiver blocks were extracted with methanol (purified by distillation from Zn-KOH). Between 1 and 2% of the total activity remained in the block. The extract was chromatographed as above with unlabeled

TABLE 53
C¹⁴-IAA TRANSPORT THROUGH 5-MM SECTIONS
OF VEGETATIVE STEM

	C ¹⁴ Donor after 3 Hours*	C ¹⁴ Tissue*	IAA-2-C ¹⁴ Receiver*	C ¹⁴ Total*
Basipetal...	1,340 $\pm$ 7.5	414 $\pm$ 8.6	76 $\pm$ 2.6	1,830
Acropetal...	1,384 $\pm$ 10.7	420 $\pm$ 7.8	26 $\pm$ 1.7	1,830

* Values are counts per minute per transport assembly and their associated standard errors of the mean.

IAA as a carrier. The R_f zone of IAA was eluted with methanol for counting. No radioactivity was detected at R_f's other than that of IAA.

Each datum presented is the mean of 3 separate experiments, each with 3 transport assemblies.

PROGRESS REPORT

Table 53 shows the distribution of radioactivity in transport experiments with sections from vegetative stem internodes. Both basipetal and acropetal transport of IAA-C¹⁴ occurred; the ratio between the two is about 3.

Table 54 shows the results of an analogous series of experiments with sections cut from stems one week after the first indication of a flowering apex. In these sections transport polarity is even less than in vegetative sections, the basipetal to acropetal ratio being about 1.3.

Both tables show a slight decrease in uptake by the vegetative tissue from the donor block when the latter is applied in an acropetal direction. Though the differences are not significant here, decreased uptakes in an

acropetal direction have also been observed by McCready and Jacobs⁽⁶⁾ for both labeled IAA-C¹⁴ and C¹⁴-labeled 2,4-D with bean petiole sections. This difference in uptake can be explained on purely physical grounds; a decrease in the concentration gradient across the gel-tissue interface would occur if the rate of transport away from that interface were reduced.⁽¹⁾

This study finds that there is no strict polarity in the longitudinal movement of IAA-C¹⁴ in either vegetative or flowering stems of *Coleus*. A similar inference based on gross bioassay methods has already been reported by Fischnich⁽¹⁷⁾ using leaf epinasty, and by Jacobs^(6, 7, 18) and Leopold and Guernsey⁽⁸⁾ using *Avena* curvature and root tests. The present work has used labeled IAA and identifies the transported radioactivity as IAA.

The rate of basipetal to acropetal transport of IAA-C¹⁴ of 3 to 1 for vegetative stem sections is similar to those found by Jacobs^(6, 7) for a different variety of *Coleus blumei*. Our observations of acropetal transport

TABLE 54
C¹⁴-IAA TRANSPORT THROUGH 5-MM SECTIONS
OF FLOWERING STEM

	C ¹⁴ Donor after 3 hours, cpm	C ¹⁴ Tissue, cpm	IAA-2-C ¹⁴ Receiver, cpm	C ¹⁴ Total, cpm
Basipetal...	1,328 ± 19.5	446 ± 18.6	56 ± 1.2	1,830
Acropetal...	1,354 ± 5.6	456 ± 13.5	43 ± 1.5	1,850

in the vegetative internode do not agree with the findings of Leopold and Guernsey⁽⁸⁾ or Haupt⁽¹⁰⁾ who reported a strict basipolarity of auxin movement in young vegetative stem sections of *Coleus*. However, our results with the flowering stem are consistent with the observation of Leopold and Guernsey⁽⁸⁾ that acropetal transport of auxin occurs in the flowering axis.

From the present experiments, it may be concluded that applied IAA itself is transported both basipetally and acropetally. It may be inferred, therefore, that the "bioassay activity" reported by Jacobs^(6, 7, 18) for vegetative stems and by Leopold and Guernsey⁽⁸⁾ for flowering stems is really derived from the transport of the added auxin, rather than being a diffusion from the tissues into the block of proximate endogenous auxin. Consistent with this view is the similarity between the ratios of transport polarities in the work of Jacobs and the present study, both using the vegetative *Coleus* stem.

CONCLUSIONS

The transport polarity of C¹⁴-methylene-labeled indoleacetic acid (IAA-C¹⁴) in stem sections of *Coleus blumei* Benth. has been examined. IAA-C¹⁴ was applied

in donor blocks of agar to one end of the tissues. Transport activity in the tissues and in receiver blocks was measured after three hours. The IAA-C¹⁴ in the receiver blocks was resolved chromatographically.

IAA-C¹⁴ was found to be transported acropetally as well as basipetally in both vegetative and flowering stem sections. A ratio of 3 between basipetal and acropetal transport rates was observed for the vegetative stem. After the initiation of flowering this ratio changed to 1.3.

REFERENCES

- Gordon, S. A. and M. Eib. Hormonal relation in the phototropic response: Polarity and velocity of auxin transport. Biological and Medical Research Division Quarterly Report, April through June, 1956. Argonne National Laboratory. ANL-5597, pp. 51-56.
- Van der Weij, H. G. Der Mechanismus des Wuchsstofftransports. Rec. trav. botan. Néerl. 29, 379-496 (1932).
- Went, F. W. and R. White. Experiments on the transport of auxin. Bot. Gaz. 100, 465-484 (1939).
- Thimann, K. V. Plant Growth Hormones. *The Hormones*, ed. G. Pincus and K. V. Thimann. Academic Press, New York, 1948.
- Cumming, B. G. The control of growth and development in Red Clover (*Trifolium pratense* L.). III. Endogenous diffusible auxin. Can. J. Botany 37, 1049-1062 (1959).
- Jacobs, W. P. Acropetal auxin transport and xylem regeneration—a quantitative study. Am. Naturalist 88, 327-337 (1954).
- Jacobs, W. P. The polar movement of auxin in the shoots of higher plants. Its occurrence and physiological significance. *Plant Growth Regulation*. Fourth Int. Conf. on Plant Growth Regulation, ed. R. M. Klein. Iowa State University Press, 1961, pp. 397-409.
- Leopold, A. C. and S. F. Guernsey. Auxin polarity in the *Coleus* plant. Bot. Gaz. 115, 147-154 (1953).
- Wickson, M. and K. V. Thimann. The antagonism of auxin and kinetin in apical dominance. II. The transport of IAA in pea stems in relation to apical dominance. Physiol. Plantarum 13, 339-354 (1960).
- Haupt, W. Gibt es Beziehungen zwischen Polarität und Blütenbildung. Ber. Deut. Bot. Ges. 69, 61-66 (1956).
- Scott, T. K. and W. R. Briggs. Recovery of native and applied auxin from the light-grown 'Alaska' pea seedlings. Am. J. Botany 50, 1056-1063 (1962).
- Stowe, B. B. Synthesis of high specific activity C¹⁴-carboxyl indoleacetic acid and of C¹⁴-nitrile indoleacetic acid. Anal. Biochem. 5, 107-115 (1963).
- Gordon, S. A. and E. Buess. Inactivity of hexitols and hexoses in biosynthesis of auxin from tryptophan. Plant Physiol. 38, 11 (1963).
- Naqvi, S. M. Transport studies with C¹⁴-indoleacetic acid and C¹⁴-2,4-dichlorophenoxyacetic acid in *Coleus* stems. Ph.D. Thesis, Princeton University, Princeton, N.J., 1963.
- Scott, T. K. and W. P. Jacobs. Auxin in *Coleus* stems: Limitation of transport at higher concentrations. Science 139, 589-590 (1963).
- McCready, C. C. and W. P. Jacobs. Movement of growth regulators in plants. II. Polar transport of radioactivity from indoleacetic acid-(¹⁴C) and 2,4-dichlorophenoxyacetic acid-(¹⁴C) in petioles of *Phaseolus vulgaris*. New Phytol. 62, 19-34 (1963).
- Fischnich, O. Über den Einfluss von β -Indolyllessigsäure auf die Blattbewegungen und die Abvektivwurzelbildung von *Coleus*. Planta 24, 552-583 (1935).
- Jacobs, W. P. The role of auxin in differentiation of xylem around a wound. Am. J. Botany 39, 201-309 (1952).

DISAPPEARANCE OF PHYTOCHROME IN OAT SEEDLINGS: KINETICS AND ACTION SPECTRUM

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PURPOSE AND METHODS

The plant pigment phytochrome is the photoreceptor for many morphogenic and biochemical processes initiated by low irradiances of visible light (see References 1 and 2 for recent summaries). Phytochrome exists in two forms, one with an absorption maximum at 660 m μ , the other at 730 m μ .⁽³⁾ These two forms of the pigment are interconverted most efficiently by wavelengths of light near their absorption maxima, a conversion that is the basis for a spectrophotometric assay for phytochrome.⁽⁴⁾ The pigment has been isolated and partially purified. It is a chromoprotein with a molecular weight between 90,000 and 150,000. The nature of the chromophore is not known.⁽⁵⁾

Phytochrome is found at relatively high concentrations in etiolated seedlings, where it occurs as the red absorbing form (P₆₆₀). Exposure to red light will convert some of the P₆₆₀ to the far-red absorbing form (P₇₃₀). It has been reported by Butler, et al.⁽⁴⁾ that either brief or continuous irradiation of the plant with red light causes the phytochrome to disappear rapidly, a disappearance ascribed wholly to destruction of the P₇₃₀. In addition, they state that some of the P₇₃₀ is transformed in the dark to P₆₆₀.

We have followed the disappearance of phytochrome and the conversion of P₆₆₀ to P₇₃₀ in etiolated oat seedlings that had been exposed briefly to white light.⁽⁶⁾ Our results suggested that both the P₆₆₀ and P₇₃₀ forms of phytochrome were destroyed, and that wavelengths other than those in the red region of the spectrum may activate phytochrome destruction. This report deals with the disappearance of phytochrome after exposure to white light and the relative action spectrum for the light-activated destruction.

Unhulled dry seeds, 10 grams, of *Avena sativa* variety Clinton were grown in plastic boxes 2.0 $\times$ 5.5 $\times$ 1.0 inches. The medium consisted of $\frac{1}{4}$ inch cellulose paper moistened with 35 ml of tap water. After sowing, the seeds were covered with a wire screen, $\frac{1}{4}$ inch mesh. The plastic boxes were placed for 5 days in light-tight boxes through which air at 23° C was passed. The plants were given the experimental exposure to light and then returned to the dark at 23°. Growth was stopped and the phytochrome stabilized by dipping the plants in ice water for about 60 seconds. Since we find that phytochrome content of intact oat shoots varies inversely with length (Table 55), only shoots 2.0 to 2.5 cm long were used for assay. The entire assay procedure was

conducted at 2–5° C. If only total phytochrome content was being determined after chilling, the assay was done under laboratory light. When assays were made for P₇₃₀ as well as for total pigment, the entire procedure was carried out under a green safe light with a transmission maximum at 565 m μ and 0.1% cut-offs at ca. 545 and 590 m μ .

The Argonne Spectrograph⁽⁷⁾ was used to obtain narrow wavelength bands for the determination of the action spectra. When necessary, light intensity was reduced by placing wire screens in the light beam emitted from the condenser fronting the spectrograph arc. The source of white light was a tungsten-filament lamp operated at a fixed voltage, different intensities being obtained by varying the distance between the lamp and the seedlings.

TABLE 55
PHYTOCHROME CONTENTS OF 5-DAY-OLD
ETIOLATED OAT SEEDLINGS OF
DIFFERENT LENGTHS

LENGTH, CM	$\Delta(\Delta O.D.)$	
	Brighton (Hullless)	Clinton
1.0–1.5.....	0.087	0.066
1.5–2.0.....	0.069	
2.0–2.5.....	0.049	0.056
3.0–4.0.....	0.039	
3.5–4.5.....		0.034

Phytochrome was determined spectrophotometrically in a dual beam spectrophotometer.^{*(3, 4)} Assay results are given in $\Delta(\Delta O.D.)$ units, which yield a relative measure of the amount of reversible phytochrome present in the plant tissue.

PROGRESS REPORT

In initial experiments etiolated seedlings were exposed to different intensities of light from the tungsten-filament lamp for 1.0 minute. They were then grown in the dark and assayed for phytochrome after various intervals up to six hours. Phytochrome contents of dark-grown controls were determined at the same intervals. The disappearance at various times, relative to the unirradiated controls, is plotted against irradiance in Figure 120.

* Ratiospect, Agricultural Research Specialty Co., Hyattsville, Maryland.

These curves show that about 25 fc-minutes saturate the light-activated disappearance of phytochrome, irrespective of the growth period following the light exposure. With 4.0 hours of growth, saturating irradiances led to a destruction of 60% of the phytochrome. No more than 60% destruction was also observed when a postirradiation growth period of 6 hours was tested.

FIG. 120.—The per cent of phytochrome that has disappeared after exposures to various intensities of light from a tungsten lamp source for 1.0 minute.

FIG. 121.—Disappearance of phytochrome after exposure to 15-foot candles for 1.0 minute (tungsten filament lamp).

In another experimental series with white light, the disappearance of total pigment, P_{730} and of P_{660} was followed. The results for all irradiances above 15 foot candles were essentially similar. Figure 121 shows the relative amount of the various forms of phytochrome after exposure to 15 fc-minutes. Immediately after the light exposure 30% of the P_{660} was converted to P_{730} . Both forms of phytochrome gradually disappeared at about equal rates. After 6 hours, no P_{730} was detected,

the total pigment concentration at this time being about 40% of the original level.

Before a detailed action spectrum was run on the light-activated destruction of phytochrome, reciprocity conditions were determined at 660 $m\mu$ and 400 $m\mu$, wavelengths found to be relatively effective in preliminary experiments. At 660 $m\mu$ incident energies of 2.3 to 8.2 $\mu W \text{ cm}^{-2}$, and at 400 $m\mu$ energies of 34.1 to 400 $\mu W \text{ cm}^{-2}$, were within the reciprocity range. The action spectrum was based on the energy required to initiate a 30% (nonsaturating) destruction of phytochrome with 4.0 hours of growth at 23° C following the light exposure. Dose-response curves using the above incident energy ranges, were run at each wavelength used; the energies required for 30% destruction were determined by interpolation. Typical curves are shown in Figure 122. For

FIG. 122.—Per cent destruction of phytochrome in 4 hours of growth following various irradiances at 600 and 660 $m\mu$.

660 $m\mu$ and 600 $m\mu$, 10 and 30 kiloergs cm^{-2} were required, respectively, in order to obtain 30% disappearance of phytochrome.

Figure 123 is a plot of the relative action spectrum for the light-activated destruction of phytochrome. There are 2 broad bands, one lying between 370 and 450 $m\mu$, the other between 550 and 700 $m\mu$ and probably peaking at about 660 $m\mu$. The red region of the spectrum is essentially 10–20 times more efficient in activating the disappearance of phytochrome. At wavelengths above 710 $m\mu$ and below 360 $m\mu$, using incident energies up to 800 kiloergs cm^{-2} , we could not obtain a 30% disappearance of phytochrome in a 4-hour growth period.

Some observations were made to determine if the conversion of P_{660} to P_{730} at several wavelengths was correlated to the amount of phytochrome disappearing. The results are summarized in Table 56. At 660 $m\mu$ there is about a 60% conversion of P_{730} and essentially the same percentage disappears. At 400 $m\mu$ the conversion to P_{730} and the loss of phytochrome increase

with irradiance at a roughly constant efficiency. Both processes show a decrease in efficiency, i.e., an indication of saturation, at the highest irradiance, 2810 kiloergs cm^{-2} . However, the percentage of phytochrome that disappears is about twice that converted to P_{730} . At 440 $\text{m}\mu$, irradiances up to 1730 kiloergs cm^{-2} yielded no conversion of P_{660} to P_{730} , yet about 50% of the phytochrome disappeared.

We have also irradiated seedlings at both 400 $\text{m}\mu$ and 660 $\text{m}\mu$ and followed this immediately with an exposure at 730 $\text{m}\mu$. The P_{730} formed at the first two wavelengths is converted equally well to the P_{660} form. Furthermore, the destruction of phytochrome induced by the first two wavelengths is partially reversed by far-red light.

We find, as do Butler et al.,⁽⁴⁾ that most of the loss of reversible phytochrome after irradiation of the etiolated plant at 660 $\text{m}\mu$ can be accounted for by the loss of the P_{730} form (Table 56). On the other hand, we find that the P_{660} form also undergoes the light-activated metabolic destruction (contrast Figure 2 with Figure 10 in Reference 1).

FIG. 123.—Incident energies required at various wavelengths for 30% destruction of phytochrome in 4 hours.

It is likely that phytochrome itself is a photoreceptor activating the metabolic self-destruction. First, the action spectrum for the process in the visible spectrum has a strong band at ca. 660 $\text{m}\mu$ and a weaker band in the region near 400 $\text{m}\mu$, with a minimum in the green. This is the complement of the *in vitro* absorption spectrum of the P_{660} form.⁽¹⁾ (The efficiencies per incident photon decrease drastically as the wavelength drops below 350 $\text{m}\mu$ [Figure 123], and no detectable destruction was observed at 300 $\text{m}\mu$. We suggest that the protein moiety of phytochrome *in vivo* does not absorb and transfer enough light energy to activate significantly the

metabolic destruction.) Second, the pigment decrease induced by irradiation at 400 $\text{m}\mu$ or 660 $\text{m}\mu$ can be partially reversed if these two irradiances are followed immediately by light at 730 $\text{m}\mu$.

However, light at 440 $\text{m}\mu$, which was ineffective in converting phytochrome to P_{730} , was effective in activating phytochrome disappearance (Table 56). This suggests that a pigment other than phytochrome might (also) function as a photoreceptor for phytochrome destruction. A lack of reversibility of the 440 $\text{m}\mu$ effect by

TABLE 56

PER CENT CONVERSION AND DISAPPEARANCE OF PHYTOCHROME AFTER IRRADIATION AT VARIOUS WAVELENGTHS AND INTENSITIES OF LIGHT

WAVELENGTH, $\text{m}\mu$	KILOERGS CM^{-2}	PER CENT CONVERTED TO P_{730}		PER CENT DISAPPEARING IN 4 HOURS	
		Observed	Per Kiloerg	Observed	Per Kiloerg
370	396	12.3	0.031		
400	468	10.8	0.023	26.2	0.056
	703	20.0	0.028	47.0	0.067
	936	30.5	0.033	56.0	0.056
	2810	38.0	0.014	62.5	0.022
440	288	0.0			
	433	0.0		18.7	0.043
	1730	0.0		47.0	0.027
660	68	59.0	0.88	61.5	0.91
	68	57.2	0.84	64.0	0.94

the far-red would be strong evidence in support of another pigment. In this connection, the "high-energy" nonreversible pigment described by Mohr⁽⁸⁾ which, has action maxima at about 440 $\text{m}\mu$ and 730 $\text{m}\mu$ should be considered. The way in which such a "blue-far red" pigment would participate is obscure, since no band in the far-red occurs in the action spectrum of phytochrome destruction (Figure 123).

Heretofore, the following relationship has been held to describe the interconversions and disappearance of the phytochrome forms:

Chiefly on the basis of the observations in this report, we would suggest the modification shown on page 202. The rate constants for the reactions indicated by asterisks are relatively low and metabolically limited.

We have used the terms "disappearance" and "destruction" with awareness of their imprecision. Since phytochrome concentration is measured by ratio spec-

trophotometry, the criterion of concentration difference is change in reversible pigment. It could well be that a drop in concentration represents a conversion of the holochrome to a nonreversible form, rather than actual disappearance of the complex.

RELATIONSHIP BETWEEN GROWTH AND METABOLISM IN SEEDS AS MODIFIED BY X-IRRADIATION

Kenneth Surrey and Edward Barr

PURPOSE AND METHODS

In the study of radiosensitivity of seeds, the most common criteria employed are the number of seeds germinated and growth indices of germinated seeds. As germination and growth are the manifestations of basic mechanism involved at the subcellular levels, the problem is to determine the physiochemical changes which are either directly involved in, or are indicative of metabolic processes of radiosensitized seeds.

Many oil-bearing seeds contain lipoxidase and also its naturally occurring substrate, linoleic acid, which is a major constituent of stored fats. In some seeds this enzyme is present in the resting stage, while in others its activity is initiated after a short period of imbibition. Whichever is the stage of its initiation, the fact that lipoxidase activity is restricted to the initial phases of seed germination makes it an ideal system to be followed as a metabolic marker during the earlier stages of seed development.

Using lipoxidase activity as the metabolic marker of the cotyledons and hypocotyl growth as an index of radiosensitivity of the embryo, the relationship of growth to metabolism in the irradiated sunflower seeds is determined.

Air-dried and dehulled sunflower (*Helianthus annuus*) seeds were given various exposures to X-irradiation. Immediately after irradiation seeds were planted in the trays. Trays were kept in the dark. Periodically seedling heights and fresh weights were determined and from this data rates of growth per day were calculated (see Figure 124).

REFERENCES

1. Siegelman, H. W. and S. B. Hendricks. Phytochrome and its control of plant growth and development. *Advances in Enzymology* **36**, 1-33 (1964).
2. Gordon, S. A. Oxidative phosphorylation as a photomorphogenic control. *Quart. Rev. Biol.* **39**, 19-34 (1964).
3. Borthwick, H. A., S. B. Hendricks, M. W. Parker, E. H. Toole and V. K. Toole. A reversible photoreaction controlling seed germination. *Proc. Natl. Acad. Sci.* **38**, 662-666 (1952).
4. Butler, W. L., H. C. Lane and H. W. Siegelman. Nonphotochemical transformation of phytochrome *in vivo*. *Plant Physiol.* **38**, 514-519 (1963).
5. Siegelman, H. W. and E. M. Firer. Purification of phytochrome from oat seedlings. *Biochem.* **3**, 418-423 (1964).
6. Chorney, W. and S. A. Gordon. Light-activated disappearance of phytochrome in oat seedlings. *Plant Physiol.* **39** (Suppl. I), 50 (1964).
7. Monk, G. S. and C. F. Ehret. Design and performance of a biological spectrograph. *Radiation Res.* **5**, 88-106 (1956).
8. Mohr, H. Primary effects of light on growth. *Ann. Rev. Plant Physiol.* **13**, 465-488 (1962).

Lipoxidase was prepared from the cotyledons (from the same lot used for growth determination), and the enzyme activities were determined spectrophotometrically.⁽¹⁾

PROGRESS REPORT

Growth of etiolated sunflower seedlings follows a typical sigmoid shaped curve: A slow increase in the initial phase (0-4 days), accelerated linear phase (4-8 days) in the second phase, and nonlinear increase thereafter. In Figure 124 are given various growth curves showing rates of growth per day calculated over linear growth phase. It can be seen that increase in X-irradiation caused a relative decrease in growth rate.

Lipoxidase activity in the cotyledons is initiated at about 36 hours, reaches its peak at about the fourth day, and then declines exponentially to the lowest level of its activity on the eighth day from the start of hydration (Figure 125, 0 kR). In irradiated seeds (10, 20, 30, 40, and 50 kR) the rates of decline decrease with increase in radiation exposures (Figure 125).

From Figure 124 it is seen that growth decreases with increase in radiation and Figure 125 illustrates that corresponding to the increase in X-ray exposures, higher levels of lipoxidase activities are maintained. Experiments were then conducted to determine growth-enzyme relation with still higher X-ray exposures. The results, together with the data presented in Figures 124 and 125, are shown in Figure 126. It may be seen that with exposures close to 50 kR, the rate of decline in enzyme activity approached zero and with still higher

FIG. 124.—Growth response curves of air-dry sunflower seeds irradiated with various exposures of X-rays. Rates of growth per day together with their standard errors, determined by least squares over the linear period (4–8 days) of growth are indicated on the respective curves.

FIG. 125.—Lipoxidase activity curves of cotyledons obtained from the seedlings of X-irradiated sunflower seeds. The rates of exponential decline in lipoxidase activity, calculated from the 5th to 8th day of the growth period together with their standard errors, are given on the respective curves.

FIG. 126.—Combined data of Figures 124 and 125 together with the responses obtained by extended radiation exposures up to 100 kR. Left ordinate denotes the rates of growth (height, ●—●; and fresh weight, ×—×), and the right ordinate is for the curve of exponential rates, ○—○.

radiation exposures the exponential rates of enzyme activity assume positive signs, indicating the elimination of activity-peaks seen in Figure 125. The exponential rate curve shows a gradual increase until about 50 kR, and thereafter (60–100 kR) it maintains close to its maximum elevation. Growth on the other hand is suppressed rapidly up to 20 kR, decreases slowly until about 60 kR, and above this dose it practically

levels off. Apparently there exists an inverse relation between growth and metabolism (Figure 126).

In order to represent growth-enzyme relation more clearly the data of Figure 126 are replotted showing the rates of growth vs. exponential rates (see Figure 127). Although there is an inverse relation between growth and enzyme activity, actual magnitudes of the relation between these two parameters differ over a different

TABLE 57
EFFECT OF X-IRRADIATION ON LIPOXIDASE ACTIVITY AND GROWTH
OF SUNFLOWER SEEDS AND COTYLEDONS

DAYS	UNITS OF LIPOXIDASE ACTIVITY/MIN/COTYLEDON								
	X-Ray Exposures, Kilorontgens								
	Intact Seeds					Cotyledons			
	100	200	300	400	500	100	200	300	400
	2.....	3.....	4.....	5.....	6.....	7.....	8.....		
2.....	9.0	5.2	0	0	0	11.0	0	0	0
3.....	39.3	19.2	14.9	0	0	23.8	3.62	0	0
4.....	51.7	25.7	24.0	7.4	0	38.3	11.8	0.76	0
5.....	70.4	48.3	38.6	14.7	0	44.8	15.1	4.79	0
6.....	74.4	54.7	43.3	15.2	0	69.1	29.1	16.7	0
7.....	81.9	67.1	44.1	16.8	0	99.6	46.4	24.3	0
8.....	87.7	69.0	45.2	18.1	0	125.3	54.6	24.0	0
Exponential Rate/Day for Lipoxidase Activity									
	0.076 ± 0.005	0.055 ± 0.009	0.022 ± 0.007	0.031 ± 0.004		0.345 ± 0.023	0.433 ± 0.056	0.523 ± 0.141	
Growth Rate, cm/day									
	0.28	0.25	0.23	0.16

FIG. 127.—Replotted data of Figure 126, showing rates of growth per day as related to the exponential rates of lipoxidase activity (cf. Figure 125).

range of X-ray exposures. For instance, from 0 to 20 kR the rate of rates of growth is greater than the one between 30 and 60 kR; this trend is, however, reversed for exponential rates of enzyme activity. The two phases are delineated by a transitional range between 20 and 30 kR. Beyond 60 kR, where the rates of growth are at a minimum, the exponential rates of enzyme activity remain close to their maximum levels.

Radiation inhibited growth, and the growth inhibition was reflected in the corresponding elevated levels of enzyme activity. Whether extended exposures to X-rays had any effect was then determined. Results of this experiment are presented on the left side of Table 57. It is shown that radiation above 100 kR impairs lipoxidase activity. Impairment not only increases with increase in X-ray exposures, but the time at which the enzyme initiation takes place is also prolonged so that above 400 kR no more enzyme activity could be detected at any stage of the sampling period.

The results reported above were obtained with dehulled intact seeds. Experiments were then undertaken to observe the metabolic responses of the dry cotyledons only. Results of this experiment are given on the right half of Table 57. Just as with the intact seeds, the metabolism of cotyledons was not inhibited up to 100 kR. Radiation of 200 kR or more decreased lipoxidase activity relative to that in the intact seeds; X-irradiation of 400 kR and above in this instance completely stopped the initiation of lipoxidase activity.

CONCLUSIONS

- 1) Growth of the embryo (as judged by the hypocotyl growth) is inversely related to the metabolism of cotyledons. However, the relationship between these two indices varies depending upon the X-ray intensities.
- 2) Variations in the relationship is dependent presum-

ably upon the degree of damage to the embryo, especially to the root meristem (as seen by the failure of primary roots to enlarge after doses above 50 kR). 3) Damage to the root meristem is reflected in growth, which in turn determines the levels of metabolism in cotyledons at various stages of seedling development. 4) Cotyledons can initiate physiological processes independently. However, their physical union with the embryo is necessary for normal seedling growth and development. 5) Embryos and cotyledons respond to X-irradiation at two different levels; normal growth process of sunflower seedlings can be inhibited to a minimal level with X-ray exposures of about 60 kR, whereas nearly seven times as much radiation is required to inhibit metabolism in the cotyledons.

REFERENCE

1. Surrey, K. Spectrophotometric method for the determination of lipoxidase activity. *Plant Physiol.* 39, 65-70 (1964).

PERMANENT RESTORATION OF SUPRALETHALLY IRRADIATED AMOEBAE BY MICROTRANSFER OF CERTAIN UNIRRADIATED SUBCELLULAR COMPONENTS

Edward W. Daniels

PURPOSE AND METHODS

The purpose of these investigations is: 1) To find precisely what components of unirradiated protoplasm have the capacity to prevent death in single cells which have been exposed to supralethal doses of ionizing or uv radiation. As a corollary, the purpose includes finding out what components of the unirradiated donor protoplasm do not possess this capacity. 2) To find which of the components of living amoebae, essential to life, is "irreversibly" damaged by radiation. The term "irreversible" is used here relative to the capacity of the exposed cell to repair lethal or supralethal radiation damage in the absence of therapeutic treatment. The restorative agent(s) might be similar to the irreversibly damaged system(s), but could conceivably be different. Three methods of attack have been used on this problem. In each of these, unirradiated protoplasm is used as a therapeutic agent, but in different ways. It is the only substance known that will prevent death in individual cells which have been exposed to supralethal doses of radiation.

Method I

Separation of subcellular components within the living unirradiated donor amoeba by centrifugal force. This produces various strata which are separated from each other by microsurgical methods. Each stratum (or combination of strata) is cut with microtools so that the plasmalemma or cell membrane remains intact. Each stratum is then injected by microfusion into a different supralethally irradiated amoeba. The theory for this method is essentially that the unirradiated component will replace and/or repair the critically damaged essential component in the irradiated cell. If replacement is the main feature of this therapy, then the donor part would probably be identical in kind to the damaged part. However, if repair is the basic process, then the damaged part might not be the same as the donor part. By this method the possible radiotherapeutically potent

components in unirradiated protoplasm have been narrowed to a relative few, and many possibilities have been eliminated.⁽¹⁻⁴⁾

Method II

This method involves the removal of unirradiated protoplasm from the donor cell into a micropipette at low temperature (1 to 2° C) and the subsequent injection of it into the supralethally irradiated cell. There are two reasons for using this method instead of Method I. First, it tells whether or not the plasmalemma is an essential part of the therapeutic agent. Secondly, it is a technique which has more potential for completely separating the various classes of restorative subcellular components for identification and study. It can be used in conjunction with the isolated strata of Method I, or it can be used separately with nonstratified cells.

Method III

This method makes use of homogenate media at low temperature (1 to 2° C) for the preparation of unirradiated protoplasm prior to its injection into supralethally irradiated amoebae. Instead of working on a single donor and a single irradiated cell at any one time, as in Methods I and II, this technique involves the homogenization of many donor amoebae at the same time. Volumes of unirradiated donor amoebae used in any one experiment range from 0.25 to 1 ml. Subcellular components can be easily washed, separated, and concentrated by this technique for later injection. In theory, it is the ideal approach to finding which class of subcellular components has radiotherapeutic capacity.

Various potential homogenate media were first studied for toxicity by microinjecting them into different amoebae. The more toxic preparations were eliminated if obvious damage was done to the recipient cell, such as swelling, vacuolization, or bursting. The least toxic media at varying concentrations and pH were further studied by noting their effects on homogenized subcellular components. Phase microscopy, electron

microscopy, and microinjection of donor homogenates into lethally irradiated cells were used to analyze the more promising homogenate media. Finally, some media were tested by grinding up the nuclei in addition to the cell in an effort to obtain radio restoration.

PROGRESS REPORT

Method I

In the past year work with this method has been limited to electron microscopic studies of stratified *Pelomyxa illinoisensis* amoebae and other species including *Pelomyxa carolinensis* and *Amoeba proteus*.⁽⁵⁾

Method II

Supralethally X-irradiated (30 kR) *Pelomyxa illinoisensis* die within a week after exposure, but death can be prevented or delayed in 85–100% of the amoebae by transfer of unirradiated protoplasm (cytoplasm + nuclei) or cytoplasm alone, by direct microfusion. We

have made many unsuccessful attempts to prevent death in these amoebae by micropipette injection of homogenates of unirradiated amoebae. To separate the causes of failure of this procedure, we attempted to transfer unirradiated protoplasm into irradiated amoebae by micropipette rather than microfusion. Thirty-six supralethally irradiated amoebae were injected with unirradiated protoplasm, isolated, and cultured, together with 47 untreated irradiated controls. Seven of the 36 treated amoebae reproduced to form mass cultures that had an average of 33 cells per clone within a month. There were no divisions among the irradiated controls. The remaining amoebae in the treated group lived significantly longer than their controls (16.0 ± 2.4 days vs. 4.6 ± 0.14 days, Student "t" test = 6.03). This work shows that unirradiated protoplasm retains its capacity for radio restoration after being removed into a foreign environment. It also demonstrates that the unirradiated plasmalemma is not an essential component for radio restoration.

TABLE 58

HOMOGENATE MEDIA TESTED ON AMOEBIA *Pelomyxa illinoisensis* IN 1964*

Homogenate Medium	Concentration and Results
Calcium chloride.....	0.16 M. Clumping of protoplasmic elements is very pronounced with calcium chloride in aqueous solution alone. Nucleus swells and nucleoli are lost although the nucleoplasm remains granular for several minutes.
Dextran.....	5% solution heated to ca. 60° C and filtered, then cooled. Intranuclear granules and nucleoli faded rather rapidly in the homogenate.
Dextrin.....	5% solution and various dilutions of it. Nuclei swell in homogenate and become washed-out. Similar to sucrose and dextrose.
Dimethyl sulfoxide.....	Intranuclear structures lost immediately as in sucrose.
Ficoll.....	Dialyzed for 1 to 5 days; 2.5 to 5% solutions tested. Nuclei in the homogenate appeared quite normal for some 20 minutes, then faded.
Glycogen.....	Dialyzed a few days; 3% about the best concentration. Nuclei in homogenate fade rather soon.
Glycogen + sodium chloride.....	3% glycogen in 0.05 M NaCl. Homogenate nuclei look normal for about 10 minutes, then fade.
Ludox (SiO ₂).....	Dialyzed 5 days in glass distilled water to a 1:1 dilution. Somewhat toxic to amoeba protoplasm; some nuclei burst, but others look quite normal for ca. 15 minutes, then fade.
Potassium chloride.....	0.16 M. Nuclei in homogenate remained granular for several minutes, but faded soon afterward.
Potassium chloride + calcium chloride....	Each compound 0.16 M in a 9:1 ratio, respectively. No significant improvement over 0.16 M NaCl. Other ratios were also tried.
Potassium chloride + sodium chloride.....	Each compound 0.16 M. Ratios 1:1, and others. No significant improvement over 0.16 M NaCl. CaCl ₂ was also introduced with these salts without apparent difference in results.
Serum (calf).....	Various dilutions, including 1:1 in glass distilled water. Some nuclei in the homogenates looked fairly normal for several minutes, but others burst.
Serum (rabbit).....	1:1 dilution in water. Some nuclei burst, but others looked quite normal with little fading for 10 minutes.
Sodium chloride.....	0.05 M to 0.5 M concentrations tested. 0.16 M was best; nuclei remained fairly normal for ca. 10 minutes.
Starch, potato (baker).....	3% dialyzed. Immediate nuclear fading.
Starch (Mallinkrodt), soluble.....	Heated (0.1 to 3%) to 75° C and cooled, then dialyzed 5 days; pH 6.6. Excellent medium for <i>Pelomyxa carolinensis</i> nuclei and other homogenate components, but the nuclei of <i>P. illinoisensis</i> faded after some 10 minutes. They lasted over an hour in <i>P. carolinensis</i> .
Starch, saturated, soluble.....	0.1% to 0.5% tested; heated to 56° C, cooled and dialyzed 4 days. Nuclei in homogenates looked good for some 10 to 15 minutes, then faded. They also slowly swelled during this time.

* Most media were in aqueous solution and in the pH range of 6.8 to 7.2, with or without adjustment with NaOH or HCl. Various concentrations of each medium were used, but only the best are tabulated here. The temperature of the homogenates was in the range of 1 to 2° C.

Method III

A number of homogenate media were previously prepared and tested in various ways.^(6, 7) This work was done at low temperature, and homogenates of the radio-sensitive amoeba, *Pelomyxa illinoisensis* were primarily studied. Some amoebae of closely related species were also used. This work was continued this year almost exclusively on *P. illinoisensis* since we found that a homogenate medium which maintained normal structures in one species of amoeba did not necessarily do the same in another. It also became clear that some media maintained relatively normal nuclei in a given species without preserving the mitochondria and vice versa. Recent work with Method III is recorded in Table 58. The data for each medium used are briefly summarized in the table. Not many of these homogenates were tested for radiorestorative capability since, in previous work,^(6, 7) if the homogenate media did not maintain normal morphological features of subcellular components, radiorestoration did not result. However, it is conceivable that the radiorestorative agent could be preserved when subcellular structures are not. Petrović has recently shown that certain homogenate fractions, particularly nuclear fractions, are restorative for irradiated mouse fibroblast cells.⁽⁸⁾

CONCLUSIONS

Much has been learned from these experiments about the nature of radiorestoration in single-cell systems. The evidence strongly indicates that unirradiated nuclei are not necessary for radiorestoration. On the other hand,

mitochondria appear to be required. Ribosome synthesis, protein synthesis, and glycolysis do not appear to be essential for radiorestoration in the sense that the exposed cell does not need new enzymes to carry out these processes. The data are not adequate, however, for the latter statement to be more than a working hypothesis. Of the three methods of approach to the problem of radiorestoration described in this report, Method II, which is the direct micropipette transfer method, and a modification of Method III, or the homogenization method, appear to have the most promise for future experiments.

REFERENCES

1. Daniels, E. W. X-Irradiation of the giant amoebae, *Pelomyxa illinoisensis*. II. Further studies on recovery following supra-lethal exposure. *J. Exptl. Zool.* **137**, 425-442 (1958).
2. Daniels, E. W. Micrurgical studies on irradiated *Pelomyxa*. *Ann. N.Y. Acad. Sci.* **78**, 662-674 (1959).
3. Daniels, E. W. and H. H. Vogel, Jr. Effects of nonirradiated protoplasm on recovery of amoebae exposed to supra-lethal doses of fission neutrons. *Radiation Res.* **10**, 584-596 (1959).
4. Daniels, E. W. and L. E. Roth. X-Irradiation of the giant amoeba, *Pelomyxa illinoisensis*. III. Electron microscopy of centrifuged organisms. *Radiation Res.* **14**, 66-82 (1961).
5. Daniels, E. W. Electron microscopy of centrifuged *Amoeba proteus*. *J. Protozool.* **11**, 281-290 (1964).
6. Daniels, E. W., E. P. Brown and Y. E. Rahman. Structural and toxicity effects of various homogenate media on subcellular components of amoebae. *J. Cell Biol.* **19**, 85 A (1963).
7. Daniels, E. W., E. P. Brown and Y. E. Rahman. Fractionation of amoebae in various media. Argonne National Laboratory Biological and Medical Research Division Seimannual Report, July through December, 1962. ANL-6790, pp., 101-112.
8. Petrović, D., B. Miletic and B. Brdar. Restoration of viability of X-irradiated L-strain cells by isologous subcellular fractions. *Intern. J. Radiation Biol.* **7**, 131-139 (1963).

PERMANENT RESTORATION OF SUPRALETHALLY IRRADIATED AMOEBAE FOLLOWING THE MICROTRANSFER OF PROTOPLASM FROM SUPRALETHALLY DRUG-TREATED AMOEBAE

Edward W. Daniels

PURPOSE AND METHODS

The purpose of these investigations is to study the biochemical nature of the radiotherapeutic agent in unirradiated protoplasm by the use of chemical inhibitors which have known sites of action in other cells. The assumption is made that these drugs attack similar sites in the amoebae under study. These experiments are designed to yield information on the critical and irreversible sites of damage in supralethally irradiated amoebae, and to improve the specificity of the therapeutic agent for this damage.

Protoplasm is transferred by the method of microfusion from lethally or supralethally drug-treated amoebae, prior to their death, into supralethally irradi-

ated amoebae.⁽¹⁾ The recipients are then cultured until death or until they reproduce many times.

The working hypothesis for this method has been, primarily, that the right chemical inhibitor or anti-metabolite, prepared in the correct manner, should irreversibly damage certain known types of essential enzymatic and/or organelle systems within the donor cell. If the lethal site of action of the drug inactivates the radiotherapeutic components of the unirradiated protoplasm, then radiorestoration following transplantation into lethally irradiated amoebae should fail, indicating that, although additional subcellular damage might also occur, the sites of radiation damage and drug action are most probably the same. However, if the

critical site of the drug action is not that of the radio-restorative component, but of some other essential protoplasmic system, then death from lethal radiation exposure should be prevented following transplantation. If cells treated in this manner recover from lethal radiation damage, the information is used as evidence that the drug-inhibited system is not essential to recovery in the supralethally irradiated cell. By using a sufficient number of the right chemical compounds, and by doing enough experiments, one should be able to find the critical sites of radiation damage which account for the death of the irradiated cells.

PROGRESS REPORT

The compounds used in this work are presented in Tables 59 and 60. The sites of their action in other cells are listed with references for each drug. Effects of these compounds on the radiosensitive amoebae under study are abbreviated in the last column of Tables 59 and 60. In general, many of the compounds listed were selected because of their affinity for the mitochondria, or for

specific reactions therein. This selection is based upon previous work in this laboratory yielding evidence which suggests that mitochondria play a vital role in radiorestitution.⁽¹⁾ Several compounds were used which inhibit reactions in the tricarboxylic acid cycle, and a number of other compounds which block electron transport at one point or another; all of these enzymatic sequences are in the mitochondria.

Still other compounds were selected which interfere with RNA and protein synthesis. These compounds were used because the need for new ribosomes in radiorestitution has not been clearly established. Electron microscopic work on the various strata in centrifuged amoebae shows that ribosomes are often scattered among the mitochondria and nuclei. Thus the influence of the ribosomes on the mitochondria in radiorestitution, if any, is unknown.

In the present work, only limited consideration was given to inhibitors of glycolysis since these enzymes are found in the cytoplasm, and since our previous investigations showed that the bulk of the cytoplasm, found in

TABLE 59
BRIEF TABULATION OF ANTIBIOTIC EFFECTS ON RADIOSENSITIVE AMOEBA, *Pelomyxa illinoisensis**

Antibiotic	Site of Action in Other Cells Reported by Various Investigators	Effects on Amoebae
Actinomycin D.....	DNA dependent RNA synthesis; inhibits messenger RNA synthesis. Nucleoli broken in kidney cells grown <i>in vitro</i> . ⁽²⁻⁶⁾	50 to 100 μ g/ml treatment for 24 hr is lethal within 1 week. Work unfinished. No transplants made.
Antimycin A.....	Inhibits cytochrome c ₁ and b; interferes with ATP synthesis. ^(7, 8)	0.2 mg/ml too concentrated and 0.1 mg/ml too dilute. Not enough margin. Work discontinued.
Aureomycin (chlortetracycline).....	Inhibits protein synthesis; mitochondria become fluorescent. ⁽⁹⁾	0.01 mg/ml treatment for 24 hr a tolerated dose (no immediate cell death). Death time too long > 2 weeks for above dose. Work discontinued.
Chloramphenicol.....	Inhibition of amino acid incorporation into protein. ^(10, 11)	2 mg/ml for 24 hr is optimum dose. About 1% of treated cells survive the treatment period. Work discontinued. Not enough cells survive treatment period.
Liquamycin (oxytetracycline).....	Inhibits protein synthesis; localizes in mitochondria. ⁽⁹⁾	0.05 mg/ml treatment for 24 hr is optimum, and this kills all cells after few days. 10 microfusions with lethally X-irradiated amoebae made. Both irradiated and drug-treated controls died at about the same time as the experimentals. Supports idea that mitochondria are essential for radiorestitution. Work is being discontinued.
Puromycin.....	Inhibition of ribosome synthesis; also amino acid incorporation into proteins. ⁽¹²⁾	0.3 mg/ml dose for 24 hr is optimum, killing all treated amoebae in a few days. From 1% to 5% of the experimentally fused cells (lethally drug-treated cells fused into lethally X-irradiated cells) survived and recovered. Many of the experimentally fused cells died immediately. Although limited in number, these data support the idea that puromycin-inhibited protein synthesis is not essential for radiorestitution. Work is being discontinued.
Terramycin (oxytetracycline).....	Inhibits protein synthesis; localizes in mitochondria. ⁽⁹⁾	0.01 mg/ml to 1 mg/ml used in 1-day treatment of amoebae. Unusually toxic for amoebae and difficult to work with. Discontinued.
Tetracycline.....	Inhibits protein synthesis; localized in mitochondria. ⁽⁹⁾	0.1 mg/ml to 1 mg/ml doses explored; 0.3 mg/ml for 24 hr is lethal dose, yet there is a time lag before death. Fusions were made between lethally drug-treated amoebae and lethally irradiated amoebae. This was not helpful, and the experimental animals died in the same time range as the treated controls. This adds to the hypothesis that the mitochondria play a vital role in radiorestitution. Work with this drug discontinued.

* Various concentrations, pH levels, and treatment times were used for each drug. A brief sampling of the literature is included in the second column.

the centripetal halves of amoebae, does not have restorative capacity if freed from the mitochondria and ribosomes.

In addition to this evidence against the need of glycolytic enzymes in radio restoration, there are data from nitrogen mustard experiments. This drug not only alkylates DNA, but it also inhibits glycolysis.⁽¹⁸⁾ The experiments on amoebae have shown that supralethal doses of nitrogen mustard do not inactivate the radio-restorative agent in unirradiated donor protoplasm. Thus, our relative neglect of glycolysis in research on the use of various chemical compounds in radio restoration seems to have been justified.

Heavy emphasis was placed upon drugs which local-

ize in the nucleus, such as colchicine, thioacetamide, and nitrogen mustard. These drugs were selected because previous work showed that the nucleus is not required for radio restoration.⁽¹⁾ The present work with lethal and supralethal doses of nitrogen mustard confirms previous findings which showed that the nuclei in unirradiated donor protoplasm are not required for radio restoration.

Cellular damage due to X-radiation is different from that caused by nitrogen mustard, Methyl-bis-(beta-chloroethyl)-amine hydrochloride (HN2), as shown by studies on the radiosensitive, giant multinucleated amoeba, *Pelomyxa illinoisensis*. All of 500 supralethally X-irradiated amoebae (30 kR) died within 8 days without dividing. Forty similarly X-irradiated amoebae were

TABLE 60
BRIEF TABULATION OF EFFECTS OF METABOLIC INHIBITORS AND OTHER CHEMICAL
COMPOUNDS ON *Pelomyxa illinoisensis* AMOEBAE*

Compound	Site of Action in Other Cells Reported by Various Investigators	Effects on Amoebae
Carbon tetrachloride.....	Endoplasmic reticulum and mitochondria. ^(13, 14)	Sat. aqueous soln., treatment 1.5 hr. Immediate toxic effects, but cells were dividing after 3 days. Work discontinued.
Colchicine.....	Mitotic inhibitor; interferes with spindle maintenance. ⁽¹⁵⁾	0.01 mg/ml to 5 mg/ml doses for 0.5 to 2 days. Lag between lethal treatment and death is long. Work unfinished.
2,4-Dinitrophenol.....	Inhibits ATP synthesis; uncouples oxidative phosphorylation; inhibits oxidation of pyruvate. ^(7, 16)	0.4 mg/ml for 18 hr killed all cells within 1 week, and 0.6 mg/ml killed all cells during the same time. 0.4 mg/ml for 17 hr allowed survivors: 30 fused (from 0.55 mg/ml grp.). Some survived in both control and experimental groups. Discontinued.
Iodoacetate.....	Inhibits ribonuclease and triosephosphate dehydrogenase (glycolysis). ⁽¹⁷⁾	$5 \times 10^{-3} M$ for 1 hr kills all but 3% of cells during treatment, but half this dose allows survivors. Work discontinued, owing to variability in successive runs.
Janus green B and Nile blue A dyes.....	Mitochondria and other sites.	10^{-3} to $10^{-6} M$ for 24 hr; lethal and nonlethal doses too close, and death follows too rapidly after lethal dose. Work discontinued.
Nitrogen mustard (HN2).	Alkylation of DNA. ⁽¹⁸⁾	See data included in text.
Sodium amytal.....	Inhibits electron transport and ATP synthesis (mitochondria). ^(7, 19, 20)	1 mg/ml to 0.05 mg/ml range of treatments each for 24-hr period. No time lag resulted after lethal dose. Discontinued.
Sodium azide.....	Inhibits oxidation of reduced cytochrome c (mitochondria); also, nuclear phosphorylation. ⁽⁷⁾	Dosage range from 0.65 mg/ml 18 hr to 5 mg/ml for 1 hr. No time lag between lethal dose and death.
Sodium cyanide.....	Inhibits cytochrome oxidase and nuclear ATP synthesis. ⁽⁷⁾	$5 \times 10^{-3} M$ for 18 hr is lethal dose, and ghosts remain. $10^{-3} M$ lethal to all of 50 amoebae except two, and these survived permanently. Work with this compound, therefore, discontinued
Sodium fluoride.....	Inhibits ATPase and enolase; (inhibits glycolysis). ⁽²¹⁾	$10^{-2} M$ too dilute to kill amoebae but $2.5 \times 10^{-2} M$ is too concentrated to produce a dependable lag between the treatment and death. Discontinued.
Sodium fluoroacetate....	Blocks aconitase (mitochondria). ⁽²²⁾	1 mg/ml for 1 day is optimum dose leaving 4% survivors which die in few days. 38 of these fused into supralethally X-irradiated amoebae. 50% of these experimentals lived significantly longer than either the irradiated or drug-treated controls. Work unfinished: all of the above experimentals eventually died.
Sodium fluoropyruvate...	Blocks step between pyruvate and acetyl Co A. (Rudolph A. Peters, Cambridge, Univ.).	Dose range: 0.1 mg/ml to 5 mg/ml. Treatment time 0.5 hr to 2 days. Several supralethally irradiated amoebae were fused with portions of lethally fluoropyruvate-treated amoebae. There was temporary vigor with cell division; longevity in the experimentals was slightly greater than in either the X-irradiated or drug-treated controls. However, all experimental animals died. Discontinued.
Thioacetamide.....	Plasmalemma loses permeability regulative capacities and allows calcium ions to enter while potassium ions are lost. Nucleoli enlarge; depression of protein synthesis. ^(15, 23)	Doses used: 2 mg/ml to 0.01 mg/ml. Treatment period = 13 hr. Nucleolar damage observed with electron microscope. Time between lethal drug treatment and death is too long. Discontinued.
Urethane.....	Has affinity for nucleoproteins; depresses respiration; causes swelling in mitochondria. ^(24, 25)	Dose range used: 0.1 mg/ml to 2 mg/ml for 1 hr; amoebae rather insensitive to strongest dose. 3 mg/ml for 24 hr recommended. Work discontinued.

* Various concentrations, pH levels, and treatment times were used for each drug. A brief sampling of the literature is included in the second column.

given unirradiated cytoplasm, without nuclei, from stock amoebae by microinjection within two days after X-ray exposure; 70% of these amoebae recovered permanently and produced cultures of many cells. This shows that the nuclei in the supralethally X-irradiated amoebae are not irreversibly damaged, and that cytoplasm of unirradiated amoebae has the capacity to prevent radiation-induced death. Each of 200 unirradiated amoebae treated for 1 hour with lethal doses of HN2 (0.1 to 0.2 mg/ml) died without division within 12 days. Similarly treated amoebae recovered and divided after injection with nucleated protoplasm from untreated stock amoebae, but not following the injection of untreated cytoplasm alone. Thus, the nuclei in lethally HN2-treated *P. illinoisensis* amoebae appear to be irreversibly damaged. Finally, 80% of 72 supralethally X-irradiated amoebae recovered permanently after microinjection with supralethally HN2-treated protoplasm which contained nuclei. Preliminary work has subsequently been done with 18 supralethally X-irradiated amoebae which received only cytoplasm from lethally HN2-treated amoebae. These organisms underwent cell division and their lifetimes were greatly extended.

CONCLUSIONS

This study will be terminated in the near future except for work with the nitrogen mustards. The latter experiments will take a few months to conclude. In general, this study with chemical inhibitors has been confirmatory to micromanipulative investigations on the problem of radio restoration. Both approaches show that unirradiated nuclei are not required for restoration after lethal radiation exposure.

Electron microscopy of restorative donor strata has shown that mitochondria are present along with some ribosomes and various small vesicles, but with very little else. This is evidence in favor of mitochondria being active in radio restoration. The work presented in Table 59, for example that on the tetracyclines, seems to indicate the same thing, namely, that the mitochondria are required for radio restoration. On the other hand, the work with puromycin suggests that the ribosomes are either not required for radio restoration, or that the lethally irradiated amoebae can reverse a significant portion of lethal puromycin damage. In any case, protoplasm from several lethally puromycin-treated amoebae prevented death in supralethally irradiated amoebae. This means that the drug did not damage, at least permanently, the radio restorative agent of donor protoplasm.

REFERENCES

1. Daniels, E. W. Recovery of reproductive function in supralethally X-irradiated amoebae following cytoplasmic microtransfer. *Progress in Protozoology*, Proc. 1st Intern. Congr. Protozool., Prague, 1961, ed. J. Ludvik et al. Czechoslovak Acad. Soc., Prague, 1963, pp. 238-239.
2. Goldberg, I. H. and M. Rabinowitz. Actinomycin D inhibition of deoxyribonucleic acid-dependent synthesis of ribonucleic acid. *Science* **136**, 315-316 (1962).
3. Goldberg, I. H., M. Rabinowitz and E. Reich. Basis of actinomycin action. I. DNA binding and inhibition of RNA-polymerase synthetic reactions by actinomycin. *Proc. Natl. Acad. Sci.* **48**, 2094-2101 (1962).
4. Reich, E. Biochemistry of actinomycins. *Cancer Res.* **23**, 1428-1441 (1963).
5. Waksman, S. A., ed. The actinomycins and their importance in the treatment of tumors in animals and man. *Ann. N.Y. Acad. Sci.* **89**, 283-486 (1960).
6. Zbarsky, S. H. The effect of actinomycin D on the incorporation of C^{14} -formate into nucleic acids of Ehrlich ascites carcinoma and Novikoff hepatoma *in vitro*. *Can. J. Biochem.* **42**, 563-566 (1964).
7. McEwen, B. S., V. G. Allfrey and A. E. Mirsky. Studies of energy-yielding reactions in thymus nuclei. I. Comparison of nuclear and mitochondrial phosphorylation. *J. Biol. Chem.* **238**, 758-766 (1963).
8. Pumphrey, A. M. Studies on the electron transfer system. XLV. Some effects of antimycin on cytochrome B. *J. Biol. Chem.* **237**, 2384-2390 (1962).
9. du Buy, H. G. and J. L. Showacre. Selective localization of tetracycline in mitochondria of living cells. *Science* **133**, 196-197 (1961).
10. Brock, T. D. Chloramphenicol. *Bacteriol. Rev.* **25**, 32-48 (1961).
11. Rendi, R. and S. Ochoa. Effect of chloramphenicol on protein synthesis in cell-free preparations of *Escherichia coli*. *J. Biol. Chem.* **237**, 3711-3713 (1962).
12. Rabinowitz, M. and J. M. Fisher. A dissociative effect of puromycin on the pathway of protein synthesis by Ehrlich ascites tumor cells. *J. Biol. Chem.* **237**, 477-481 (1962).
13. Bain, J. A. and S. E. Mayer. Biochemical mechanisms of drug action. *Ann. Rev. Pharmacol.* **2**, 37-66 (1962).
14. Reynolds, E. S., R. E. Thiers and B. L. Vallee. Mitochondrial function and metal content in carbon tetrachloride poisoning. *J. Biol. Chem.* **237**, 3546-3551 (1962).
15. Buer, D. J. Effects of colchicine and thioacetamide on amoebae. Argonne National Laboratory Semester Student Project Report 1-31 (1964).
16. Eisenhardt, R. H. and O. Rosenthal. 2,4-Dinitrophenol: Lack of interaction with high-energy intermediates of oxidative phosphorylation. *Science* **143**, 476-477 (1964).
17. Lamden, M. P., A. P. Mathias and B. R. Rabin. The inhibition of ribonuclease by iodoacetate. *Biochem. Biophys. Res. Commun.* **8**, 209-213 (1962).
18. Wheeler, G. P. Studies related to the mechanisms of action of cytotoxic alkylating agents: A review. *Cancer Res.* **22**, 651-688 (1962).
19. Chance, B. and G. Hollunger. Inhibition of electron and energy transfer in mitochondria. I. Effects of amytal, thiopental, rotenone, progesterone and methylene glycol. *J. Biol. Chem.* **238**, 418-431 (1963).
20. Ernster, L., G. Dallner and G. F. Azzone. Differential effects of rotenone and amytal on mitochondrial electron and energy transfer. *J. Biol. Chem.* **238**, 1124-1131 (1963).
21. Rice, L. I. and D. A. Berman. Malonate and fluoride effects on metabolism and contraction of electrically stimulated heart strips. *Am. J. Physiol.* **200**, 727-731 (1961).
22. Adams, E. Poisons. *Sci. Am.* **201**, 76-84 (1959).
23. Barker, E. A., E. A. Smuckler and E. P. Benditt. Effects of thioacetamide and yellow phosphorus poisoning on protein synthesis *in vivo*. *Lab. Invest.* **12**, 955-960 (1963).
24. Isenberg, H. D., J. I. Berkman and L. H. Sundheim. The response of *Potriochromonas stipitata* to urethan and related compounds. *J. Protozool.* **9**, 40-44 (1962).
25. Mookerjee, S. and M. G. Alamelu. Nuclear cytoplasmic separation in N-ethyl urethane treated amoebae. *The Nucleus* **2**, 215-222 (1959).

THERAPY OF POISONING BY RADIOACTIVE AND NONRADIOACTIVE METALS

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PURPOSE AND METHODS

The purpose of this project is the development and evaluation of new concepts and techniques for the therapy of poisoning by radioactive and nonradioactive metals, particularly those depositing in bone. An integral part of the program is the development of a better understanding of the mechanisms of deposition, translocation, and toxicity of these substances in tissues. Two therapeutic approaches have received major attention. The first involves the use of chelating agents, e.g., diethylenetriaminepentaacetic acid (DTPA), which have a high formation constant with the toxic metal relative to competing metal cations normally present in the body. Compounds of this type are effective in the removal of metals such as plutonium and lead. The second aims at manipulation of normal physiological processes to accelerate excretion of the toxic metal.

Plutonium, a prototype of the heavy, polyvalent, easily hydrolyzable radioelements which polymerize and form colloidal and insoluble aggregates under physiological conditions, has received particular attention. The measurement of these colloids and the effect of their formation on the tissue distribution, ease of removal by chelation therapy, and on long-term effects of retained radioelements are continuing problems.

The efficacy of potential therapeutic agents and their mechanisms of protection are investigated by a) study of the alteration of the median lethal dose in acute metal poisoning, b) observation of the effects of tissue distribution and excretion by means of radioactive assay, autoradiography, and ultrafilterability changes and c) assessment of the effects on chronic metal poisoning in terms of changes in tumor incidence, histopathology, and life expectancy.

PROGRESS REPORT

Measurement and Control of Colloidal Properties of Plutonium

In Solutions.—An ultrafiltration technique which characterizes the colloidal state of a solution of plutonium by measuring the proportion of plutonium filterable through a cellophane membrane has been used to study some of the parameters responsible for the colloidal properties of plutonium in aqueous solutions.⁽¹⁾ In the course of this work procedures were worked out for the preparation of reasonably reproducible solutions of monomeric plutonium. Subsequently, a need arose for the preparation of $\text{Pu}(\text{OH})_4$ particulates of approxi-

mately 0.1–10.0 microns in diameter. It was found possible to prepare such particulates by regulation of the molar excess of citrate to 10, or lower, and careful upward adjustment of pH. Ultrafiltration of the supernates produced in these procedures also yielded a long-sought clue to the preparation of plutonium solutions of reproducibly low ultrafilterability (a problem hitherto beyond our technical ability). The preliminary conclusion is that the aggregation of $[\text{Pu}(\text{OH})_4]_n$ is limited by a small excess of citrate ions which compete with hydroxyl ions for the Pu^{+4} cation.

In Vivo.—For dosimetry and for experimental therapeutic purposes quantitative information on the fractionation of plutonium deposited in the organic and the hard portions of bone is needed. Such information is needed, for example, to determine the function of the reticuloendothelial elements of the bone marrow in the phagocytosis of radiocolloids. Chemical separation of the marrow and its associated plutonium from the hard bone has thus far proved to have limited usefulness.⁽²⁾ A physical separation technique has now been used to measure the differential distribution of plutonium in the marrow and on the bone surface of a standard segment of tibial shaft 6 and 90 days following administration to mice of three plutonium solutions of varying colloidal properties. This experiment, involving radiochemical and autoradiographic analysis, is being conducted in cooperation with J. Marshall and J. Farnham of the Radiological Physics Division. The colloidal properties of the plutonium solutions were measured by the ultrafiltration technique and by the differential uptake of plutonium in the tissues of the mouse.

The plutonium analyses of tissues from mice killed at 6 days showed a different pattern of deposition for each of the three solutions. Injection of a highly colloidal solution (Pu 18% ultrafilterable) was followed by a liver deposition of 80% of the injected dose (I.D.) and a bone deposition of 12.6% I.D. The intermediate colloidal plutonium solution (Pu 30% ultrafilterable) was followed by a deposition of 46% and 24.4% I.D. in liver and bone, respectively, and the monomeric solution (Pu 90% ultrafilterable) by 28% and 42.4% I.D. The analyses of the tibial shaft segments showed that about $\frac{1}{5}$ of the plutonium in the segment was deposited in the marrow after the highly colloidal solution, $\frac{1}{4}$ following the intermediate colloidal solution, and only $\frac{1}{20}$ following the monomeric solution. The rapid filtration of the highly colloidal form of plutonium by the liver presum-

ably reduced the amount of this form available for deposition in the bone marrow. The differences in distribution in the liver and bone marrow emphasize the importance of the reticuloendothelial system in the initial uptake of the hydrolyzable elements which are commonly referred to as bone-seekers.

Intraskelatal Distribution of Plutonium

A common assumption in estimating uptake of a radioelement in bone is that total skeletal content = content in both femurs $\times 10$, based on early findings in the rat. Questions arose concerning the validity of this assumption in the mouse receiving different colloidal forms of plutonium and treated with DTPA. An experiment was performed with monomeric plutonium to determine the variations in the amount of plutonium deposited in different parts of the skeleton, with and without DTPA therapy, and to correlate the specific activity of the bone with the location of plutonium-induced bone tumors. Results are as follows: a) Optimal DTPA therapy removed monomeric plutonium from each bone to about the same degree. b) The femur is the most representative bone in terms of uptake of plutonium. The femurs represented 7.6% of the total skeletal ash and contained 8.3% of the retained plutonium, in the case of the control mice, and 7.8% in the case of the DTPA-treated mice. In no other bone did the ratio of bone weight to bone plutonium more closely equal one. c) On a weight basis alone the factor of 13 is more exact than the factor of 10 in computing mouse skeletal burden from the content of both femurs. On the basis of the measured plutonium content of the total skeleton, the factor was closer to 12.5, both with and without therapy. This factor was also found to hold for colloidal plutonium.

DTPA Therapy in Plutonium Poisoning

Long-Term Effects.—Two experiments have been undertaken in mice to a) correlate the long-term effects of deposited plutonium, primarily the induction of malignant bone tumors, with the skeletal burden of plutonium and b) determine the long-term effects of DTPA therapy. In the first experiment, in which a colloidal form of plutonium was injected, optimal therapy initiated at 3 days removed one-half of the plutonium deposited in the bone and reduced the incidence of mice with bone tumors by one-half.⁽³⁾

The second experiment, in which injection of monomeric plutonium was followed by optimal DTPA therapy at 3 days or at 1 hour has been described and the results of treatment on removal of plutonium summarized.⁽⁴⁾ All the mice in this experiment are now dead, and the following summary can be made. The plutonium

level in the bones of mice treated with DTPA given at 3 days and 1 hour was reduced to $\frac{1}{2}$ and to $\frac{1}{4}$ of the control level, respectively. Life expectancy was correlated with the amount of plutonium retained. Mean survival time of plutonium controls was 361 days; that of DTPA-treated groups 450 and 557 days, respectively; that of mice receiving only DTPA was 650 days. Similarly, for each group the latent period (time to the death of the first mouse with a bone tumor) increased with the amount of plutonium removed by therapy—from 153 days in the plutonium controls to 236 and 354 days in the two treated groups. The rate of death with bone tumors was slower in the DTPA-treated groups. The final proportion of mice with bone tumors was 82% in the control group receiving monomeric plutonium, compared with 73% in the 3-day treated group and 60% in the 1-hour treated group. Only the latter reduction is significant.

Thus, in contrast to the results following colloidal plutonium, reduction in bone tumors in mice receiving monomeric plutonium was not proportional to the amount of radioelement removed from bone by DTPA therapy. Removal of one-half of the plutonium from bone had no effect on final bone tumor incidence, while reduction of the skeletal burden to one-quarter of the control level (achieved by initiating therapy before skeletal deposition was complete) reduced the tumor incidence only to three-quarters of that of untreated mice. The effects of DTPA therapy on the life-time development of bone tumors, therefore, depend upon the form in which the plutonium is administered. The data also indicate that monomeric plutonium is more effective than colloidal plutonium in the production of malignant bone tumors.

Modification of DTPA To Improve Cellular Uptake.—Since DTPA remains essentially extracellular, it is unable to chelate with and remove intracellular deposits of plutonium which, in the case of a colloidal form, may represent a considerable fraction of the body burden. Presumptive increase in the cellular permeability of DTPA was achieved by the use of the more lipid-soluble pentaethyl ester of DTPA. Therapy with the DTPA ester was followed by removal of a fraction of colloidal plutonium unavailable to the calcium chelate of DTPA.⁽⁵⁾ Another approach to the problem of increasing intracellular localization of DTPA is the production of a polymerized DTPA molecule to promote its uptake by the phagocytic cells which also phagocytize colloidal plutonium. So far, preliminary attempts to produce the polyethyl ester of DTPA by condensation of the acid form with ethylene glycol in the presence of concentrated sulfuric acid have not yielded a product identifiable by chemical analysis or infrared spectrum as the polyester.

Development of New Therapeutic Approaches

Part of this program is the evaluation of new drugs and different forms of therapy in radioelement poisoning. We have recently tested an organic chelating agent desferrioxamine B-sulfonate (DFOM), derived from Actinomycetes, against plutonium both by the ultrafiltration screening technique and in mice injected with plutonium.⁽⁶⁾ DFOM has been used clinically in patients suffering from iron overload. On the basis of published stability constants, DFOM would be expected to be more effective in binding plutonium than iron. At approximately equimolar concentrations of chelating agent and plutonium, DFOM proved more effective than DTPA in increasing the ultrafilterability of plutonium in blood plasma; at higher concentrations (molar ratio = 10), DFOM was less effective than DTPA. In spite of the greater effectiveness of DFOM at low concentrations *in vitro*, it was found to have limited usefulness in removing plutonium from the mouse. Optimal DFOM therapy initiated 3 days after administration caused a small increase in urinary excretion (about 4% of the injected dose) and prevented further uptake of plu-

tonium by the bone. It was not able to remove any of the radioelement that had been deposited in the skeleton.

REFERENCES

1. Lindenbaum, A. and W. M. Westfall. Colloidal properties of plutonium in dilute aqueous solution. Intern. J. Radiation Isotopes, in press.
2. Markley, J. F., M. W. Rosenthal and A. Lindenbaum. Progress report: Plutonium removal. VIII. Chemical removal of marrow and its associated plutonium from bone. Argonne National Laboratory Biological and Medical Research Division Semiannual Report, January through June, 1963. ANL-6823, pp. 109-110.
3. 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).
4. Rosenthal, M. W., J. F. Markley and A. Lindenbaum. Progress report: Plutonium removal, tumor incidence studies. IV. Argonne National Laboratory Biological and Medical Research Division Semiannual Report, July through December, 1962. ANL-6790, pp. 87-91.
5. Markley, J. F. Removal of polymeric plutonium from mice by combined therapy with the calcium chelate and pentaethyl ester of DTPA. Intern. J. Radiation Biol. 7, 405-407 (1963).
6. Rosenthal, M. W. and A. Lindenbaum. Effect of desferrioxamine B-methane sulfonate (DFOM) on removal of plutonium *in vitro* and *in vivo*. Proc. Soc. Exptl. Biol. Med. 117, 749-750 (1964).

DATA PROCESSING

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PURPOSE AND METHODS

The two main activities of the Data Processing Section are the operation of a machine complement and the development of effective techniques for handling data. The section has had a major revision during the past year. The IBM 1620 computer, which had 20,000 decimal digits, has been replaced by a GE 225 computer with 8,192 twenty-bit words. The complement includes a high speed printer, four tape drives, an auxiliary arithmetic unit, and an on-line plotter.

Miss Carol Vancina of the Data Processing Section and GE Applications Engineering Personnel assisted in revising the program library and establishing more effective data handling procedures.

PROGRESS REPORT

The computer activities can be divided into three groups.

Self-programming Activities

To aid the people writing their own programs an elementary course in Fortran programming was offered by Mr. Robert Hartman of General Electric. Six people are now engaged in writing their own programs, and all programs of this nature which had been written for the IBM 1620 have been converted to the new system.

Statistical Programs and Programs for Which the Scientist Maintains His Own Data

A list of the programs which have been converted and documented or are in the process of being documented are given below. For convenience, some of the programs exist in several forms, but are only listed once.

- a) Least square $s = 1 - (1 - e^{-KD})^n$.
- b) Linear regression—with transforms and 95% confidence limits.
- c) Gompertzian $w = w_0 e^{A/a(1-e^{-at})}$.
- d) Probit—with weighting factors and 10, 20, 30, . . . 90 centile points.
- e) One-way analysis of variance with means and "t" test.
- f) Linear regression—fixed slope.
- g) Simultaneous equations.

- h) Eigenvalues—eigenvectors.
- i) Fisher's exact method.
- j) Per cent protein globulin calculations.
- k) Multivariate analysis of variance.
- l) Generalized multiple linear regression, covariance and correlation with transforms.
- m) Generalized Fortran plotter subroutines.
- n) Bibliography program.

Larger Programs—Data Remains in Custody of the Data Processing Section

- a) Soil and air analysis (Gustafson). The soil and air calculations have been converted and a gamma-ray spectrum program written.
- b) Mouse irradiation and genetics (Grahm). The data from this set of experiments have been maintained and continuously up-dated.
- c) Dose survival studies (Stearner). The programs to make the 30-day mortality spread and to compute per cent mortality have been converted.
- d) Dog program (Norris). The basic data handling, tapes, and programs have been completed to make, up-date, copy, and revise. A program has been written to calculate and recover the weights at 100, 200, and 300 days. Preliminary runs have been made on the census program.

Special Interest.—The following activities deserve special attention.

- a) Radiation effects. Through close work with S. P. Stearner and S. A. Tyler, a program has been developed to fit the growth of chickens to the model $w = w_0 e^{A/a(1-e^{-at})}$ and a paper presented⁽¹⁾ on the effects of radiation on growth at the Radiation Research Society meeting. In this paper it was shown that if growth is assumed to follow the above model where A , a , and w_0 are parameters of growth, A and a are essentially dose independent and w_0 is dose dependent.
- b) Bibliography. A program has been designed to keep a bibliography of the literature published by the members of the division. The papers are listed by four classifications: open literature, abstracts, ANL literature, and books. The program is capable of listing the complete bibliography, the bibliography by author, or the bibliography by year. Included in the program is a tape-write and an up-date feature. The main advantage of this program is to lessen the problem of handling the published material.

Summer Program.—As part of our program six summer students spent considerable time in computer work. The three who worked closely with the Data Processing Section were:

- a) Miss Sylvia Tyler (Fisk University) revised and updated a program concerning the rodent colony for Dr. Flynn.
- b) Miss Karen Johnson (Lawrence University) developed

several statistical programs including a least squares fit of the logistic function.

- c) Miss Mary Ellen Steverson (University of Illinois) developed a generalized multiple linear regression and adapted several programs to the new system.

REFERENCE

1. Stearner, S. P., S. A. Tyler and M. H. Dipert. The influence of gamma radiation on growth. *Radiation Res.* 22, 240 (1964).

DYNAMICS OF CELLULAR POPULATIONS

Ernesto Trucco and George A. Sacher

PURPOSE AND METHODS

It is desired to find a mathematical formalism that will adequately describe the changes occurring in cellular systems, particularly self-renewing cell populations.

Methodologically, the first step consists in partitioning the population into demes. A deme includes the totality of cells answering a well-defined (but incomplete) state description. The changes referred to above, therefore, will be known if the characteristics specifying a deme, i.e., its "constitution," can be predicted as a function of time. In the simplest case the constitution of a deme is sufficiently well determined by its age distribution.

Problems of this nature were studied long ago by A. J. Lotka in a different context (the evolution of biological systems). A highly sophisticated mathematical tool, developed in recent years, provides, in principle, the most adequate description of events occurring within a deme. This is the theory of branching processes⁽¹⁾ which has a wide range of applications (including, for example, the study of cosmic-ray showers). At any instant of time a cell may select one of several alternatives, such as dividing, ceasing to exist, or migrating to another deme, in accordance with certain probability laws; thus, the number of cells in a deme becomes a stochastic, or random, variable. The theory of branching processes is designed for situations of this kind. A simpler version of the probabilistic theory, dealing with expected values only, was outlined by H. Von Foerster.⁽²⁾ We have extended his work, giving a more rigorous formulation of his results. A first attempt to apply the theory for the case of the leukopoietic system is also being made and is dealt with at length in the report on Computer Simulation of Granulocyte Kinetics.

In previous studies^(3, 4) we used a slightly different though related approach, quite similar in its principal aspects to the early work of Lotka.

PROGRESS REPORT

A model of recovery from radiation injury, based on essential characteristics of cellular populations, and a method for obtaining the length of life distribution in the erythrocyte population of small mammals, are described in the report on the Stochastic Theory of Mortality.

A paper⁽⁵⁾ now in press contains a detailed study of the Van Foerster equation, and gives some examples illustrating the formalism. According to this theory a deme is completely specified by its age density function; this, in turn, depends on, a) the initial age distribution in the population, b) the rate at which cells of age zero are fed into the deme, either by cell division or by immigration from precursor populations, and c) a quantity called the loss function which must be determined experimentally. Various extensions of Von Foerster's equation are now being investigated by D. G. Oldfield in his thesis written at the University of Chicago. The introductory section of Reference 5 serves to clarify the concept of deme which was proposed some time ago by one of us (G. A. S.). It is suggested that in the future the term "compartment" should be used only for aggregates that may be regarded as homogeneous with respect to all the properties of their constituents. This requirement is fulfilled, for example, by the compartments of chemical tracer kinetics.

In the simple models studied so far the population consists of a single deme whose loss term, λ , is a function of age only—an assumption which is sufficient for many practical applications. Similar problems have been treated by several authors, notably Harris, Bellman, Kendall, and Waugh, under the name of age-dependent branching processes (for a summary of this work, see Reference 1, chapter VI). It can be shown that an integral equation derived by Harris for the expected value of the population size also follows from Von Foerster's formalism. On the other hand, the variance and the

higher moments of the population size cannot be obtained from the Von Foerster equation. In certain important cases of interest to us, i.e., the turning-over cell populations in the organism, the estimates of second and higher order moments provided by the theory of branching processes do not reflect the true state of affairs. This is because the experimentally observed fluctuations are preponderantly due to variations in the system parameters, as opposed to effects of random sampling in a system with fixed parameters. However, for some special situations (e.g., the first steps of tissue regeneration from a very small number of cells) the stochastic element may acquire primary importance, and a deterministic treatment would be inadequate.

Actually, self-renewing cellular systems include several demes, so that one Von Foerster equation must be written for each of them. In general, the birth or loss functions of a deme depend on the events occurring in other demes ("feedback interaction"), and so we have to deal with a system of interrelated equations. Then it becomes exceedingly difficult to obtain explicit solutions. We conclude, therefore, that machine computations are indispensable in most simulation studies, such as the one on granulocyte kinetics mentioned above, and others that can be found in the literature.⁽⁶⁾

CONCLUSIONS

The work performed has shown the usefulness of the Von Foerster equation for a theoretical treatment of problems in the field of cellular population dynamics. Recent papers, e.g., Stanners and Till⁽⁷⁾ or Puck and

Steffen,⁽⁸⁾ and the experiments on cell cultures by El-kind, Sinclair, and others can also be better analyzed and understood in terms of the Von Foerster equation. It provides a framework which is simpler to handle than the stochastic theory of branching processes, and yet sufficiently general as a guide and tool for the interpretation of results obtained by experimental physiologists.

REFERENCES

1. Harris, T. E. *The Theory of Branching Processes*. Springer, Berlin, 1963.
2. Von Foerster, H. Some remarks on changing populations. *The Kinetics of Cellular Proliferation*, ed. F. Stohlman, Jr. Grune and Stratton, New York, 1959, pp. 382-407.
3. Sacher, G. A., E. Trucco and J. H. Borrowman. Mathematical models for the formation and regulation of populations of formed blood elements; I. Some basic concepts and a first specific model. Argonne National Laboratory Biological and Medical Research Division Semiannual Report, July through December, 1958. ANL-6093, pp. 46-54.
4. Sacher, G. A. and E. Trucco. Progress report: Dynamics of cellular populations. Argonne National Laboratory Biological and Medical Research Division Semiannual Report, July through December, 1959. ANL-6264, pp. 52-54.
5. Trucco, E. Mathematical models for cellular systems; The Von Foerster equation. Parts IA and IB. *Bull. Math. Biophysics*, in press.
6. Warner, H. R. and J. W. Athens. An analysis of granulocyte kinetics in blood and bone marrow. Leukopoiesis in health and disease. *Ann. N.Y. Acad. Sci.* 113, 523-536 (1964).
7. Stanners, C. P. and J. E. Till. DNA synthesis in individual L-strain mouse cells. *Biochim. Biophys. Acta* 37, 406-419 (1960).
8. Puck, T. T. and J. Steffen. Life cycle analysis of mammalian cells; I. A method for localizing metabolic events within the life cycle, and its application to the action of colcemide and sublethal doses of X-irradiation. *Biophys. J.* 3, 379-397 (1963).

STOCHASTIC THEORY OF MORTALITY

George A. Sacher and Ernesto Trucco

PURPOSE AND METHODS

The general ideas underlying the stochastic theory of mortality and the aims to be achieved are described in previous publications.^(1, 2) The mathematical investigation that has been performed using the Fokker-Planck equation⁽³⁾ practically exhausts the possibility of a purely analytic treatment. Quite some time ago, therefore, it was decided that only a Monte Carlo simulation procedure could yield further results of interest.⁽⁴⁾ Since then, various possibilities have been examined.^(4, 5) As we had anticipated, the problem of writing a program suitable for our purposes turned out to be exceedingly complex. The development of the Monte Carlo program is now being handled by Dr. D. A. Woodward of the Applied Mathematics Division. Progress was reported by him both on conceptual and on technical points.

However, it is expected that some time will elapse before we can have a running program. The calculations will be performed on the Applied Mathematics Division's CDC-3600 computer, taking full advantage of the increased capabilities afforded by that machine.

PROGRESS REPORT

While efforts toward an adequate Monte Carlo program continue to be a major theme of our investigations, three other aspects closely related to the general theory of mortality have been actively pursued.

Factors Governing Longevity of Mammals

One of us (G. A. S.) further developed his studies on the interrelation between brain weight, body size, energy expenditure, and longevity. New data were analyzed, confirming previous findings and hypotheses.

The most interesting aspect of this work is the positive correlation between index of cephalization and length of life which was obtained in a great variety of mammalian species. The index of cephalization is taken as a measure for internal control and information handling capacity of the organism. As a result of this analysis the emphasis is gradually being shifted from a theory of mortality *per se* to a theory of vertebrate longevity: it seeks to determine the extent to which longevity, mortality, and the rate of aging depend on the capacity of the organism for control and adaptation of its physiological performances. This point of view was presented in two symposium papers^(6, 7) given at meetings of the American Psychological Association and the Operations Research Society of America.

Radiation Mortality and Recovery from Radiation Injury

This work is based on two hypotheses, namely: 1) A set of plausible equations—called the recovery equations—specifying the laws of growth of tissue cell populations as well as the effects of ionizing radiations on cell survival and reproduction; 2) The assumption that death of the organism occurs when the number of cells in a critical tissue is depressed below a certain criterion value.

At present the theory is still deterministic, but the stochastic element will eventually be introduced when the Monte Carlo program is working. The recovery equations are chosen in accordance with known or inferred properties of cellular systems. Hence, this phase of our study is also related to the project "Dynamics of Cellular Populations." So far, two simple cases have been investigated in detail. First, a one-compartment system whose dynamic behavior is governed by the Pearl-Verhulst logistic equation. This model was examined for two kinds of irradiation: continuous exposure, on one hand, and two single doses with a variable time interval between them ("split dose technique") on the other hand. The equations derived in the former case have been used to fit some experimental data of S. A. Tyler and S. P. Stearner.⁽⁸⁾ The second model introduces two cellular compartments, "proliferative" and "functional," without feedback interaction (which is certainly an unrealistic assumption). The results obtained from this system for the case of continuous exposure agree in some respects with an experimental curve recently published by Sacher and Grahn.⁽⁹⁾ Serious discrepancies remain, but these are due to factors not yet taken into account by the model, including feedback control, finite maturation time, and aging processes.

The work done thus far concludes the first part of this study. It has been reported at the 1964 Radiation Re-

search Society meeting, and will be the subject of a paper that should soon be ready for publication. The computer work, including the preparation of several graphs with a machine plotter, was done by Mrs. Ruth Fu of the Applied Mathematics Division.

Life-Span of Erythrocytes in Small Mammals Determined from Whole-Body Counts of Radioactivity

Total-body counts were recorded daily for five months from mice which had received Fe^{59} -labeled erythrocytes from an isologous donor. Counts were also recorded from mice which had been given one intravenous injection of a Fe^{59} solution at time zero. From the moment when a radioactive red blood cell disintegrates, the Fe^{59} released to the blood plasma will suffer the same metabolic fate as radioactive iron injected directly into the blood stream. The mathematical formulation of this statement leads to a Volterra integral equation of the first kind. The kernel and the known function can be obtained from the experimental data; the unknown function is the lifetime distribution of erythrocytes which we seek to determine. This can be found by an iterative numerical computation performed on a digital computer. An algorithm for the solution of Volterra integral equations, which had been developed by Dr. D. L. Phillips of the Applied Mathematics Division, was used to compute the erythrocyte survival distributions. The results obtained so far are sufficient to demonstrate the feasibility of the method. The first phase of this work (in particular the gathering of data) is nearly completed, and a report will soon be in preparation. It should be possible, by these methods, to measure the survival of red cells in small animals subjected to different treatments, e.g., X-irradiation.

CONCLUSIONS

Work on the Monte Carlo program must be completed as a preliminary to our main effort on the stochastic theory of mortality. The other studies mentioned in this report have been partially completed, with some degree of success, but they have not yet reached their final stage. A fully integrated theory stressing the stochastic and the system-theoretic aspects of the life and death process still remains the long range objective of this project.

REFERENCES

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

- theory of mortality. I. and II. *Bull. Math. Biophys.* 25, 303-323 and 343-366 (1963).
4. Sacher, G. A. and E. Trucco. Monte Carlo calculations for the stochastic theory of mortality. Argonne National Laboratory Biological and Medical Research Division Semiannual Report, January through June, 1962. ANL-6723, pp. 65-66.
 5. Sacher, G. A. and E. Trucco. Progress report: Monte Carlo calculations in the stochastic theory of mortality. Argonne National Laboratory Biological and Medical Research Division Semiannual Report, July through December, 1962. ANL-6790, p. 20.
 6. Sacher, G. A. The evolution of longevity as an organized behavior: dependence on energy metabolism and brain structure. *Proc. Am. Psychol. Assoc. Mtg.*, Los Angeles, September 7, 1964. To be published.
 7. Sacher, G. A. Dimensional approach to the relation of brain structure to behavior: application to the theory of longevity. Paper presented at the meeting of Operations Research Society of America, Honolulu, September 15, 1964.
 8. 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).
 9. Sacher, G. A. and D. Grahn. Survival of mice under duration-of-life exposure to gamma rays. I. The dosage-survival relation and the lethality function. *J. Natl. Cancer Inst.* 32, 277-320 (1964).

KINETICS OF RADIATION INJURY ACCUMULATION AND REVERSAL

S. Phyllis Stearner, Sylvanus A. Tyler, and Margaret H. Sanderson

PURPOSE AND METHODS

The kinetics of injury accumulation during irradiation is of special interest in the study of radiation damage. Also of concern is the manner in which injury is reversed after the end of an exposure. Study of the kinetics of these processes has led to speculations concerning the possible nature of the effects involved in the lethal response. An empirical model describing the course of mortality in terms of dose and exposure time was developed for acute radiation effects in birds.⁽¹⁻³⁾ This model was subsequently applied to mortality data for the mouse.⁽⁴⁾ Acute radiation lethality was postulated to be the arithmetic sum of lethality due to reversible and irreversible processes, the former dependent on and the latter independent of exposure time. In this context, time-independent or irreversible processes refer to those mechanisms that are invariant with exposure periods up to one day only and are expressed as lethality some time within 30 days after exposure. It was further postulated that the injury associated with each effect accumulates independently and that death of the irradiated animal occurs when the critical level (lethal bound) for either type of effect is exceeded. The data are consistent with the assumption that loss of dose effectiveness for time-dependent effects occurs at a constant fixed rate. Thus, this rate is not influenced by the size of the radiation dose or the amount of accumulated injury. In several strains of mice (including data from other investigators) there is a constant relation between radiosensitivity and reversal rate. That is, an amount of radiation equal to the D_0 for 50% mortality* is reversed in the same amount of time in all strains of mice for which data are available. In addition, comparison of dose reduction kinetics in three species of

bird for which we have survival data indicates that the exposure-time effects, normalized for differences in instantaneous dose sensitivity, are comparable.⁽⁴⁾ However, the relative reversal rate, based on a D_0 of unity, is faster in the bird than in the mouse by a factor of two. It would seem, then, that the reversal rate during irradiation, at least for the indicators under consideration, is an inherent characteristic of the individual animal and is related to its ability to survive radiation injury. The greater the radiosensitivity (i.e., the smaller the D_0), the smaller is the reversal rate during exposure. The manner in which the radiation effect is reversed during an exposure, for the time period to which we are limiting ourselves, is as yet undetermined. However, it may be that the reversal involves some repair at the subcellular level rather than cell proliferation.

A related question concerns the kinetics of dose reduction (injury reversal) after the end of an exposure. In the mouse, comparison of dose reduction during and after the end of an exposure (protracted vs. split exposures) shows that the reversal kinetics are in good agreement through the first four to six hours.⁽⁵⁾ Reversal after the end of an exposure appears to be limited to this time period and there is no further dose reduction within the 24-hour period under consideration in these studies.

On the basis of a linear model of dose reduction during and after irradiation, early reversal after the end of an exposure (within 24 hours) should occur at a rate that is independent of the size of the first dose.

This point has been discussed at some length by several investigators. It is generally held that the reversal rate is exponential and proportional to the size of the first dose. In some instances the time periods exceed 24 hours, and comparisons are based on different intervals. Where investigations include shorter periods (<24 hours), results are frequently equivocal or contradictory, usually because of insufficient sample size.

* The notation D_0 refers to the amount of radiation which, if delivered instantaneously, will result in the designated effect—in this case 30% mortality.

PROGRESS REPORT

We have experiments under way which are designed to evaluate the effect of the size of the first dose of a split exposure on the rate or amount of dose reduction (reversal) within 24 hours after the first exposure. Initial investigations are on the LAF₁ mouse. Preliminary results tended to confirm the predictions of the model, but the experiment could not be satisfactorily completed because of an outbreak of *Pseudomonas* in the stock colony. The infected animals gave results that were in no way comparable to previous data accumulated over a 5-year period. Thus, these experiments have been temporarily discontinued. Meanwhile, a split-dose exposure study was initiated on the 3-day chick and is now under way. First doses of 300 and 700 R are used, and intervals range up to 24 hours. At each interval a series of second doses is used, adjusted to cover the lethal range. As an experimental animal for radiation mortality studies, the chick has many advantages

over the mouse. It does not suffer from the many diseases that plague the mouse and has provided reproducible results in our experiments for more than 15 years. This split-dose experiment utilizing the 3-day chick is now in progress, and results will be available for analysis during the coming year.

REFERENCES

1. S. P. Stearner 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).
2. S. A. Tyler and S. P. Stearner. Discrimination among injury processes reflected in acute radiation mortality. *Intern. J. Radiation Biol.* 14, 495-509 (1962).
3. S. A. Tyler 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. S. P. Stearner 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.* 20, 619-630 (1963).
5. S. A. Tyler and S. P. Stearner. Accumulation of acute radiation injury associated with split and fractionated doses. *Radiation Res.* 21, 16-25 (1964).

COMPUTER SIMULATION OF GRANULOCYTE KINETICS

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PURPOSE AND METHODS

Some years ago, H. M. Patt and M. A. Maloney, then working in this Division, gave a single injection of tritiated thymidine to dogs (beagles) and carefully measured the number of labeled neutrophil granulocytes in the circulating blood over a period of time ranging from approximately 90 to 150 hours after injection of the label. This was done by taking hourly blood samples from which autoradiographs were prepared with AR-10 stripping film. The fraction of labeled cells, based on 1,000 cells from each slide, was determined. Two slides were counted by two independent observers for each experimental point. In this way, graphs showing the percentage of circulating labeled neutrophils vs. time were obtained. Moreover, the tagged cells were subdivided into four classes according to their grain count: 1) cells with 4-9 grains, 2) with 10-19 grains, 3) with 20 or more grains, and 4) total number of cells labeled with at least 4 grains, i.e., the sum of categories 1, 2, and 3.

It was felt that a computer simulation might be useful in trying to understand and interpret the curves found by Patt and Maloney; more generally, a study of

this nature would provide better insight of the granulopoietic system as a whole. In addition to the unpublished material mentioned above, many other results obtained in several years of work have been or will be used in this investigation. For example, Figure 2 of Reference 1 shows the percentage of labeled precursor cells of the myeloid series found in the marrow after a single injection of tritiated thymidine. However, most of the information needed in setting up the computer program is summarized in two basic papers by H. M. Patt and M. A. Maloney,^(2, 3) which contain the experimental data underlying their model of granulocytopoiesis.

As a first step in our program the nonproliferative (i.e., maturation and functional) part of the system was considered. At present, therefore, the computer model includes only four demes.† These are, a) metamyelocytes, b) band cells, c) segmented neutrophils, also called mature polymorphonuclear granulocytes ("polys"), all of them in the marrow, and d) mature polys in the blood.

PROGRESS REPORT

For a mathematical formulation of the problem it was found convenient to use the equation first derived

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‡ As explained in Reference 4, the word "deme" is preferable to the more usual term "compartment" for cellular populations.

by H. Von Foerster (cf. the report on Dynamics of Cellular Populations). This work, however, already involves a generalization of the original Von Foerster equation because we are now dealing with populations of labeled cells. Hence, in addition to cellular age, the grain count obtained from a cell in the process of autoradiography was taken as a second property characterizing demes.

A paper⁽⁵⁾ now in press indicates briefly the relevant steps of the calculation. For each deme the Von Foerster equation yields relations between the influx of labeled cells and either the outflow or the percentage of labeled cells present in the deme, as a function of time. The same equations were also derived by means of simple hydrodynamic models of fluid flow through a compartment (R. N. Buchal, unpublished). Without going into details we can state that the mathematical analysis is greatly simplified by the following circumstances: a) no cell division occurs in the demes under consideration, and b) the total population (labeled plus unlabeled cells) is assumed to be in a steady state.

The input-output relations were programmed on a digital computer in order to simulate the behavior of labeled and unlabeled cells under various assumptions, specified by the parameters appearing in the loss function of each deme. It is hoped, eventually, to match the experimental curves by appropriate choice of those parameters, but at present only preliminary results are available. However, an important and interesting consequence of the theory developed so far is R. N.

Buchal's "area rule," which states that the time integral of certain quantities (taken from time zero to infinity) should have the same value for each deme.

The computer program was written, and is currently being operated, by A. J. Strecok, of the Applied Mathematics Division. We wish to express our thanks for his very valuable assistance and cooperation.

CONCLUSIONS

The work done so far has resulted in a better understanding of the problems involved in our study, and this, in turn, has enabled us to complete the preliminary investigations leading to a concrete program for the digital computer. The program itself is only in its beginning stage, so that the few results obtained from it are not significant and certainly not conclusive. Further experimental work, now being performed at the University of California, is needed in order to determine the model system unambiguously.

REFERENCES

1. Patt, H. M. and M. A. Maloney. Kinetics of neutrophil balance. *The Kinetics of Cellular Proliferation*, ed. F. Stohman, Jr. Grune and Stratton, New York, 1959, pp. 201-207.
2. Patt, H. M. and M. A. Maloney. An evaluation of granulocytopoiesis. *Cell Proliferation*, ed. L. F. Lamerton and R. J. M. Fry. Blackwell, Oxford, 1963, pp. 157-171.
3. Patt, H. M. and M. A. Maloney. A model of granulocyte kinetics. *Ann. N.Y. Acad. Sci.* 113, 515-522 (1964).
4. Trucco, E. Mathematical models for cellular systems; the Von Foerster equation; Part 1A. *Bull. Math. Biophys.*, in press.
5. Trucco, E. Mathematical approaches to the study of cellular populations. *Proc. Second Annual Symposium on Biomathematics and Computer Science in the Life Sciences*, in press.

ESTIMATION OF GENERATION TIME AND DIVIDING FRACTION OF A CELL POPULATION LABELED WITH TRITIATED THYMIDINE

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PURPOSE AND METHODS

The experiment and hypotheses that form the background of this investigation are as follows. Mammalian cells of a single type are "flash" labeled *in vivo* with tritiated thymidine. Thymidine is a specific precursor of DNA. Within 30 minutes following injection, thymidine is either incorporated into DNA or catabolized to nonutilizable products. Only cells synthesizing DNA take up thymidine. The DNA is metabolically stable, and thus the thymidine incorporated into DNA of cells is stable; and, if radioactive, the radioactivity per cell is decreased only by cell division or physical decay. The latter is negligible in that the half-life of the label is 12 years. The labeled cells are autoradiographed and the number of silver grains above each cell is counted (called the "grain count") and is assumed proportional

to the radioactivity present in the cell. It is further assumed that the population of cells under consideration is asynchronous and equivariant (equal generation time) for which

1. The total number of cells increases with time, and
2. Cell death and cell migration are negligible.

The specific question with which we are confronted is this: How can an estimate of the generation time and size of a subpopulation of dividing cells be determined despite the presence of nondividing or limited-dividing cells?

PROGRESS REPORT

First, we consider a cell population in which all cells are dividing. The population is exposed to the label and on division the grains of a labeled cell are partitioned

with equal probability between the 2 daughter cells according to the binomial law. Both labeled and unlabeled cells are scored. Let the grain count distribution of the initially observed population be represented by the probability function

$$\rho_0(i) = p_i; \quad i = 0, 1, 2, \dots, R;$$

$$\sum_{i=0}^R p_i = 1 \quad (1)$$

where $\rho_0(i)$ is the probability that a cell of this population contains i grains and R is either finite or infinite. The mean and variance of this distribution are

$$\mu_0 = \sum_{i=0}^R i p_i \quad \text{and} \quad V_0 = \sum_{i=0}^R i^2 p_i - \mu_0^2. \quad (2)$$

It can be shown that

$$\mu_k = \mu_0 \left(\frac{1}{2}\right)^k, \quad V_k = \left(\frac{1}{4}\right)^k \{V_0 + (2^k - 1)\mu_0\} \quad (3)$$

and

$$\rho_k(x) = \left(\frac{1}{2^k}\right)^x \sum_{i=x}^R p_i \binom{i}{x} \left(1 - \frac{1}{2^k}\right)^{i-x}, \quad (4)$$

where μ_k , V_k and $\rho_k(x)$ represent the mean, variance, and probability distribution of grains among daughter cells

at time kT after observing the initial population. The time T is the constant generation time.

Next, we construct a cell population possessing the feature that among daughter cells of the k^{th} generation a constant fraction $(1 - a)$ of them fails to divide again. These cells remain in the population and have a grain count distribution similar to the distribution of a totally dividing population at time kT . It has been shown that the mean M'_t of this mixed population at any time t after observing the initial population is given by

$$M'_t = \frac{(1 - 2a)\mu_0}{1 - a[1 + (2a)^{t/T}]}. \quad (5)$$

Estimates of a and T can be determined using Expression 5 by employing nonlinear regression techniques on the observed set of paired values, (t, M'_t) . A consequence of Expression 5 is that for $a < \frac{1}{3}$, the mean grain count will never halve.

This model has been further extended by utilizing the more realistic assumption that the label is attached to the chromosomes of the cell and that the chromatids associated with each chromosome are independently partitioned between daughter cells. A similar procedure was derived for estimating a and T from the observed relationship between time and mean grain count.

MECHANISMS OF INJURY ACCUMULATION UNDER ACUTELY LETHAL IONIZING RADIATIONS

Sylvanus A. Tyler and S. Phyllis Stearner

PURPOSE AND METHODS

A reduction in dose effectiveness of ionizing radiations in producing acute lethality is supported by experimental results on a wide variety of organisms. However, idealized descriptions of the manner in which this reduction takes place are considerably different.⁽¹⁾ The most widely accepted concept states that the rate of reduction is proportional to the lethal injury accumulated (exponential hypothesis).⁽²⁾ From the results of acute experiments with chicks, parakeets, pigeons, and mice,^(3, 4) we were led to postulate that the rate of reduction is essentially constant (linear hypothesis). The magnitude of this constant is an inherent characteristic of each individual organism. The purpose of this study is to test the validity of these hypotheses. LAF₁ mice were subjected to fast, split doses of CO⁶⁰ γ rays separated by intervals of a few minutes to 6 hours.

PROGRESS REPORT

Three first (conditioning) doses were given—300 R, 700 R, and 900 R. The first two doses given alone

were not expected to produce any acute mortality, whereas, 900 R was found to produce approximately 40% mortality within 30 days of the exposure. If reduction in dose effectiveness is proportional to the lethal injury sustained, then one would expect the magnitude of the reduction to be an increasing function of the conditioning dose. If the linear hypothesis is upheld, then the same reduction should be observed irrespective of conditioning dose employed. Statistical analyses of results of the split dose experiment suggest that the linear hypothesis is tenable. The experimental results are not conclusive since a significant proportion of the mice was found to be infected with a radiosensitive pathogen. Plans to repeat this experiment are under way.

The 900 R experiment is of special interest in that this dose alone produces considerable mortality. It is usually the custom among experimenters to avoid delivering a first dose that can produce a significant proportion of acute deaths. The reason is that a dose of this magnitude introduces an additional complication into

the observed patterns of mortality. However, split dose experiments with conditioning doses in the lethal range may prove valuable in testing not only the constancy of the reduction in dose effectiveness, but also the independence of the several injury mechanisms that are operative as a consequence of the irradiation. Analysis of results for the 900 R experiment showed that if the mortality observed subsequent to the second dose was corrected for the expected decedence of the conditioning dose alone, both the independence assumption and the constant dose-reduction assumption seem feasible.

THE EFFECT OF PROTRACTED DOSES ON GROWTH

Sylvanus A. Tyler, S. Phyllis Stearner, and Merlin H. Dipert

PURPOSE AND METHODS

Our objective in pursuing this study was to devise mathematical procedures by which to assess the effects of dose and exposure configuration on the normal growth pattern of mammals and investigate the similarities in growth behavior between strains and species. Since the most extensive data readily available were collected from the chicken,⁽¹⁾ detailed analysis was begun with birds.

Leghorn chicks that survived acute irradiation with X-rays and γ rays and comparable controls were weighed at frequent intervals until death. More than 300 birds are included in this observation, and survival ranged from 1 to 10 years. With X-rays, only fast single doses were given. With γ rays, the total dose was either delivered within 60 minutes or protracted over 24 hours. Total doses ranged from 700 to 2200 R. This spread of dose covers the acute lethal range.

PROGRESS REPORT

First, a mathematical description of the normal growth pattern was sought; and for this first approach, only the initial rapid phase of growth was considered. This phase of growth was described by the set of linear differential equations

$$\begin{cases} \frac{dw}{dt} = \gamma w \\ \frac{d\gamma}{dt} = -\alpha \gamma \end{cases} \quad (1)$$

REFERENCES

1. Quastler, H. Time-dose relations in radiation effects. *Proceedings of Conference on Research on the Radioltherapy of Cancer*. American Cancer Society, New York, 1961, pp. 100-112.
2. Blair, H. A. On addition of modes of radiation injury in producing lethality in dogs and rodents. University of Rochester Atomic Energy Project Report UR-654 (1964).
3. Stearner, S. P. and S. A. Tyler. Radiation mortality in the mouse: Model of the kinetics of injury accumulation. 1. Protracted doses in the 30-day lethal range. *Radiation Res.* 20, 619-630 (1963).
4. Tyler, S. A. and S. P. Stearner. Accumulation of acute injury in the mouse subjected to split or fractionated doses of X-radiation or gamma radiation. *Radiation Res.* 21, 16-25 (1964).

The solution of Expression 1 is Gompertz' equation:

$$W(t) = W(0) \left\{ \text{Exp} \frac{A}{a} [1 - \text{Exp}(-\alpha t)] \right\}, \quad (2)$$

where $W(t)$ is the weight of the organism at time t , and A and α are constants. During the year, we have accomplished the following:

1. Constructed a computer program, adapted for the GE225 computer, that produces weighted least squares estimates and standard errors of A , α , and $W(0)$ for the set of ordered pairs $[t, W(t)]$;
2. Computed these constants for each member of the experience;
3. Plotted the observed weight curve of each member.

Analyses of the statistical results suggest that

1. The constants A and α are not appreciably affected by dose and exposure time,
2. The estimated initial weight $W(0)$ (the weight at hatching in this case) decreases as dose is increased,
3. Protraction lowers the rate of decrease of $W(0)$ with respect to dose.

If one considers the initial weight of an unirradiated control as related to "critical mass" upon which subsequent growth depends, then radiation could be thought of as an agent that reduces that mass.

REFERENCE

1. Stearner, S. P. Unpublished observations.