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ORNL-969
Progress Report

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BIOLOGY DIVISION

QUARTERLY PROGRESS REPORT

for Period Ending February 15, 1951

Alexander Hollaender, Editor

DATE ISSUED MAY 21 1951

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ORNL-644	Period Ending February 15, 1950
ORNL-727	Period Ending May 15, 1950
ORNL-807	Period Ending August 15, 1950
ORNL-889	Period Ending November 10, 1950

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TABLE OF CONTENTS

INTRODUCTION 5

PRESENTATION OF RESEARCH RESULTS TO THE SCIENTIFIC PUBLIC 7
- Publications 7
- Scientific Society Lectures and Traveling Seminars 9
- Visiting Lecturers 10

CYTOGENETICS 11
- Cytogenetic Effects of Radiation (Paramecium) 11
- Effects of Radiation on Rate of Mitosis 20
- Effects of Radiation on Microorganisms 23

MAMMALIAN GENETICS AND DEVELOPMENT 33
- Genetic and Developmental Effects of Radiation in Mice 33

PATHOLOGY AND PHYSIOLOGY 36

MICROBIOLOGY 41
- Tracer Studies on Metabolism 41

BIOCHEMISTRY 46
- Studies on Nucleic Acids, Enzymes, and Energy-Transfer Systems 46
- Radiobiochemistry 59
- Plant Physiology 62

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INTRODUCTION

Review of research progress. The special group set up for the investigation of protective action of chemicals on bacteria has studied a variety of groups of compounds, four of which have been found to effect striking modification of X-ray damage in these organisms. These were compounds containing (1) sulfhydryl groups, (2) certain alcohols and glycols, (3) redox compounds, and (4) certain metabolic intermediates and products. Considerable practical importance might be attached to these findings if they should also be found to protect mammals against X-ray damage. Tests for this possibility are now in progress. It appears that this protection is active not only against toxic effects but also against mutation production.

Analysis, on the basis of metabolic systems, of the radiation effects of X rays on the rate of mitosis is an aim of one group of investigators. Preliminary results showing a variety of effects on *Paramecium* have been obtained with our new X-ray machine.

The investigation of the effects of an acute dosage of X rays on mutation production in mice has been extended to give highly significant results in regard to mutation rate. Data obtained so far justify the statement that the X-ray-induced mutation rate in mice is considerably higher than that in *Drosophila* for at least one type of mutation in small, but similarly selected, samples of loci. These data may constitute a warning that estimates of human hazards based on *Drosophila* mutation rates may be too low by about one order of magnitude. These findings are very important in establishing safe limits of X-ray exposure.

Continued study of the effects of slow neutrons has brought out some interesting data on leukemias. A conspicuous difference in the effectiveness of cobalt gammas as compared with 250 kv X rays has been found in connection with cataract production. These results are similar to those discovered in peanuts after the same treatments. Further data have been obtained on the diversion of red blood cells into the lymph after exposure to X rays. A long-term study of the effect of C^{14} on a variety of functions in mice is well under way.

Further interesting results have been obtained on the analysis of breakdown products of nucleic acids. The intelligent use of the ion-exchange methods employing different means of breaking down the nucleic acids has given a whole spectrum of new compounds.

Separation of a number of sugars has been accomplished by the Plant Physiology group. They have been able to follow the labeling with C^{14} in some of these sugars.

Another lecture series on radiological physics has been started by the Radiobiochemistry group. The effect of varying the oxygen tension of erythrocytes on their sensitivity to radiation-produced potassium loss has been studied and it has been found that the oxygen tension has no effect on this function.

A new problem has been initiated wherein careful analysis is made of the combined effects of infrared and X rays on *Tradescantia* microspores. The object of this analysis is elucidation of the possible mechanisms involved in chromosome damage by this combination of rays. As previously observed, the combined X rays and infrared will produce considerably more damage than X rays alone, while infrared alone produces no recognizable effects.

The work with tracers in intermediary metabolism has progressed so successfully that it is planned to make this field the major topic of the Biology Research Conference to be held in 1952.

PRESENTATION OF RESEARCH RESULTS TO THE SCIENTIFIC PUBLIC

Publications. The following papers by members of the Biology Division have appeared in the open literature during the quarter. Only two reports have been published in the ORNL literature.

AUTHOR (S)	TITLE OF PAPER	OPEN PUBLICATION
W. T. Burnett, Jr., R. R. Bigelow, and C. W. Sheppard	Radiomanganese Studies in the Pancreatic Fistula Dog (Abstract)	Am. J. Physiol., 163:702, 1950
S. F. Carson	Organic Acid Synthesis by C_2 Condensation; Reversible CO_2 Exchange Reactions (Abstract)	Brookhaven Conference Report BNL-70 (C-12), 56-63, 1950
W. E. Cohn and C. E. Carter	The Separation of Adenosine Polyphosphates by Ion Exchange and Paper Chromatography	J. Am. Chem. Soc., 72:4273, 1950
J. Furth, G. A. Andrews, R. H. Storey, and Leon Wish	The Effect of X Irradiation on Erythrogenesis, Plasma and Cell Volume	Sou. Med. J., 44:85, 1951
J. Furth	Experimental Radiation-Induced Hormone-Secreting Ovarian Tumors, Adenocarcinoma with Hypervolemia	V. Congres International du Cancer, p. 131, July, 1950
A. Hollaender, George Stapleton, and F. L. Martin	X-Ray Sensitivity of *E. coli* as Modified by Oxygen	Nature, 167:193, 1951
J. C. Kile, Jr.	An Improved Method for the Artificial Insemination of Mice (Abstract)	Anat. Rec. 108:557, 1950
H. I. Kohn	Changes in the Plasma of the Rat During Fasting; the Influence of Genetic Factors Upon the Sugar and Cholesterol	Am. J. Physiol., 163:410, 1950
G. R. Noggle	The Use of Isotopes in Soil Research	Scientific Monthly, LXXII 1951
L. B. Russell and W. L. Russell	The Effects of Radiation on the Preimplantation Stages of the Mouse Embryo (Abstract)	Anat. Rec., 108:521, 1950
C. W. Sheppard	The Disturbance by X Rays of Selective Potassium Accumulation in Human Erythrocytes (Abstract)	The Biol. Bull., 99:310, 1950
Gordon W. Walker and Walter S. Wilde	The Exchange of Potassium in Plasma, Liver and Muscle (Abstract)	Am. J. Physiol., 163, 759, 1950
Maurice Whittinghill	Some Effects of Gamma Rays Upon Recombinations and on Crossing Over in *Drosophila Melanogaster* (Abstract)	Genetics 35:140, 1950

PROJECT
PUBLICATIONS

Maurice Whittinghill	Some Effects of Gamma Rays Upon Recombinations and on Crossing Over in Drosophila Melanogaster	ORNL-817
Edited by A. Hollaender	Biology Division Quarterly Progress Report, Period Ending November 10, 1950	ORNL-889

Collection and editing of ten papers given at our 1950 Biology Conference on Biochemistry of Nucleic Acids, along with the open discussion on these papers and an additional paper by Dr. W. E. Cohn of this Division, have been completed. This material is in press for publication as a Supplement to the Journal of Cellular and Comparative Physiology under the title "Symposium on Biochemistry of Nucleic Acids." Following is the table of contents.

Introduction	Alexander Hollaender
Organic Chemistry of Nucleic Acids	A. M. Michelson
Some Results of the Application of Ion-Exchange Chromatography to Nucleic Acid Chemistry	Waldo E. Cohn
Some Recent Studies on the Composition and Structure of Nucleic Acid	Erwin Chargaff
On the Nature of the Products Formed by the Action of Crystalline Ribonuclease (Kunitz's Ribonuclease) on Yeast Ribonucleic Acid	Gerhard Schmidt, Ricardo Cubiles, and S. J. Thannhauser
The Distribution and Interrelation of Nucleic Acids in Fixed Cells as Shown by Enzymatic Hydrolysis	Berwind P. Kaufmann, Margaret R. McDonald, and Helen Gay
Studies on the Desoxypentose Nucleic Acid Content of Animal Nuclei	Arthur W. Pollister, Hewson Swift, and Max Alfert
Precursors of Nucleic Acids	George Bosworth Brown
Biosynthesis of the Purines	John M. Buchanan
The Origin and Metabolism of Ribose	Dwight B. McNair Scott and Seymour S. Cohen
B-Vitamins and the Biosynthesis of Purines and Pyrimidines	William Shive
Microbiological Systems Involving Nucleic Acid Derivatives	Helen R. Skeggs

Scientific society lectures and traveling seminars. Activity in this phase of research has been steadily mounting during the quarter and will reach its peak during the spring quarter. Oral presentations given are listed.

W. A. Arnold	University of Illinois Urbana	Light Production in Plants
W. A. Arnold and W. G. Stone	Association Southern Agricultural Workers Memphis	Stable Isotopes
W. A. Arnold	N. E. Tenn. Section Am. Chem. Soc.	Problems Connected With Photosynthesis
Alan Conger	Emory University Atlanta	Cytogenetic Effects of Slow Neutrons from an Atomic Reactor
Alan Conger	Emory University Atlanta	Effect of Radiation on Chromosomes
J. Furth and K. W. Christenberry	National Res. Council Meeting on Radiation Cataracts Washington, D.C.	Cataract Induction in Mice by Slow Neutrons and Gamma Rays
Mary E. Gaulden	University of Florida Gainesville	Effects of Radiation and Chemical on Cell Structure and Mitosis as Revealed in the Living Cell
Mary E. Gaulden	Emory University Atlanta	Same Subject
Mary E. Gaulden	University of Georgia Athens	Same Subject
Alexander Hollaender	Meharry Medical College Nashville	Studies on the Biological Effects Radiation
Alexander Hollaender	University of Tenn. Med. School Sigma Xi Memphis	Factors Modifying Sensitivity of Living Cells
Alexander Hollaender	AAAS - Symposium on Biological Effects of Radiation Cleveland	Secondary Factors Influencing Sensitivity of Living Cells to Radiation
J. C. Kile, Jr.	AAAS - Soc. of Am. Zool. Cleveland	An Improved Method for the Artificial Insemination of Mice
G. R. Noggle	Assoc. Sou. Agri. Workers, Plant Physiol. Section Memphis	Application of Radioactive Isotopes to Plant Physiological Research
L. B. Russell and W. L. Russell	AAAS - Soc. Am. Zool. Cleveland	The Effects of Radiation on the Preimplantation Stages of the Mouse Embryo

Representatives of the Biology Division who will present papers at the Federation of American Societies for Experimental Biology, which convenes in Cleveland, Ohio on April 29 are Dr. C. W. Sheppard (coauthor of paper, Miss

Marian Stewart), Dr. B. L. Strehler (coauthor, Dr. W. A. Arnold), Dr. K. W. Christenberry (coauthor, Dr. J. Furth), Dr. Waldo E. Cohn, Dr. W. T. Burnett, Jr. (coauthors, Dr. A. Hollaender and Mr. G. E. Stapleton). Mrs. Nyra Harrington will present a paper, coauthored by R. W. Koza, at the Association of Southeastern Biologists in Tuscaloosa, Alabama on April 20-21. Two papers will be given at the Southeastern Section of the American Physical Society in Chattanooga, April 5-7, one by Dr. C. W. Sheppard (coauthor, Dr. L. J. Savage) and the other by Edgar B. Darden, Jr. (coauthor, Dr. C. W. Sheppard). Later in the season there will be additional offerings at the Society of American Bacteriologists, American Association for Cancer Research, American Chemical Society, and other meetings.

Visiting Lecturers. It is the policy of the Biology Division to bring in scientists of established reputation for lectures and consultation. The following seminars have been given during this quarter by speakers from other laboratories.

Dr. Gerhard Schmidt - The Boston Dispensary - "Recent Trends in the Biochemistry of Nucleic Acids"

Dr. Sterling B. Hendricks - USDA, Bethesda - "Photoperiodism"

Dr. George O. Gey - Johns Hopkins Hospital - "Comparative Observations on Living and Malignant Cells"

Dr. Kenneth B. Raper - USDA, Northern Regional Research Laboratory - "Cellular Organization and Differentiation in Simple Slime Molds"

Dr. C. S. Shoup - Division of Biology, AEC, Oak Ridge - "Studies in Limnology"

Dr. A. D. McLaren - Brooklyn Polytechnic Institute -"Photochemistry of Enzymes and Viruses"

Dr. L. C. Dunn - Columbia University - "Mutations Affecting the Axial System in the Mouse"

Dr. Samuel P. Hicks - Peter Bent Brigham Hospital - "Some Effects of Ionizing Radiation on the Developing Mammalian Nervous System"

Dr. L. O. Krampitz - Western Reserve University - "Mechanism of Acetate Formation of Bacteria"

Dr. Darwin L. Wood - University of Michigan - "The Infrared Microspectroscopy of Living Muscle Cells"

CYTOGENETICS

CYTOGENETIC EFFECTS OF RADIATION (PARAMECIUM)

R. F. Kimball (Leader)
A. D. Conger
Seymour Pomper
A. H. Doermann
Betty B. Hill
Nenita Gaither
Mary Kathryne King
D. S. Daniels
Lucille M. Fairchild
Kathryn M. Daniels

Effects of various environmental circumstances on reduced vigor after autogamy. (Kimball, Gaither, King) As has been brought out in previous reports (detailed presentation in ORNL-174 and ORNL-175), irradiated paramecia when sent through the sexual process of autogamy produce many clones which are not viable or multiply at a slow rate. Such reduced vigor has been interpreted as being due to gene mutations made homozygous by autogamy. Previous reports have brought out that the frequency of clones of reduced vigor with a given dose of ionizing radiation is subject to modification as follows. (1) Irradiating the animals late in the division interval has less effect than irradiating early (ORNL-174). (2) Irradiating in the presence of oxygen is more effective than irradiating in its absence (ORNL-889: 12-16). (3) Photoreactivation does not occur for X-ray-induced reduced vigor although it does for that induced by ultraviolet (ORNL-806).

During the present quarter two other circumstances have been investigated in some detail: (1) starvation prior to irradiation, and (2) temperature during the 24 hours immediately after irradiation. Since the condition of the animal (stage of the division cycle) had already been shown to have considerable effect, it appeared worth while to investigate the effect of starvation. Temperature during the first 24 hours after irradiation has been shown (ORNL-244: 18-28) to have a marked influence upon recovery from radiation-induced division delay in *Paramecium*. While *a priori* no effect would be expected on induced gene mutations, it appeared worth while to investigate the matter in some detail, especially as the question has been raised whether radiation-induced reduced vigor is entirely due to mutational changes. The results of both the temperature and starvation experiments are shown in Table 1. The well-fed animals used for comparison with the starved animals had gone through division about 1 hour before irradiation. The animals were irradiated at room temperature; a number of irradiated animals were isolated, placed immediately

TABLE 1

Absence of effect of temperature and starvation upon reduced vigor induced by X rays

EXPERIMENT	TEMPERATURE (°C)	FEEDING	DOSE OF X RAYS (r)	NUMBER OF LINES	REDUCED VIGOR (%)
50.47	31	well fed	1700	12	26.0
	26.5	well fed	1700	12	31.7
	20	well fed	1700	12	20.3
	15	well fed	1700	12	17.0
50.45	31	well fed	3300	15	70.4
	26.5	well fed	3300	15	60.5
	20	well fed	3300	15	65.1
	15	well fed	3300	15	61.1
50.49	26.5	starved	2000	17	17.6
	26.5	starved	4000	17	52.2
	15	starved	2000	17	20.0
	15	starved	4000	17	43.0
50.50	31	well fed	3100	20	60.2
	31	starved	3100	20	42.8
	26.5	well fed	3100	20	53.8
	26.5	starved	3100	20	49.6
	9	well fed	3100	20	54.4
	9	starved	3100	20	50.2
51.3	26.5	well fed	5300	20	51.6
	26.5	starved	5300	20	69.6

at the desired temperature, and kept there for 1 day before return to the usual 26.5° C. The percentages of reduced vigor given in column 6 are based upon twenty-five autogamous clones from each of the lines of column 5. The percentages for each line were transformed to angles by the arcs in tranformation and analyses of variance carried out for each experiment separately. No significant differences between any of the treatments were found. Thus it can be concluded that starved animals respond to X rays just as do well-fed animals in the early part of the interdivision interval and that the temperature after irradiation is without effect upon the changes which lead to reduced vigor.

Reduced vigor after autogamy following high doses of X rays. The availability of the Maxitron-250 machine on a routine basis during this quarter has made possible a beginning of work upon the effects of high doses of X rays. Some time has been spent in measuring the unfiltered beam with a nylon 250-r Victoreen thimble chamber. At 10.5 cm from the target, the intensity is approximately 33,000 r per minute as measured by this instrument. Time has also been given to designing a method for exposure of the animals in thin layers. The design has been completed, but work with it has not yet started. However, preliminary data have been obtained from exposures under less ideal conditions.

The results for reduced vigor after autogamy are shown in Fig. 1. For comparison, a curve is shown for experiments with the Maxitron-250 in which a 3-mm Al filter was used. Taken at their face value the points suggest a maximum at about 20,000 r followed by a small minimum at about 40,000 r with an asymptotic approach to 100 per cent at approximately 100,000 r. Powers (ANL-4333: 127-134) has reported a similar secondary minimum, though higher doses, for another strain of this same species. However, the secondary minimum in the present data is based upon a rather small sample and could represent nothing more than a sampling fluctuation. The failure of the curves for the unfiltered and filtered beams to overlap may well be due to difficulty in making comparable measurements of these beams and also to the less than ideal exposure conditions used in these preliminary experiments.

Delay in cell division by X rays. The availability of high-intensity source has made possible a start on investigation of division delay by X rays. Investigations with ultraviolet-induced delay have lead, after reconsideration, to the adoption of the reciprocal of the time to the sixth division as the best single measure of the effect. This reciprocal represents, of course, the rate for this group of six divisions to occur.

Dosage relations are shown in Fig. 2 in which the reciprocal of the time to the sixth division is plotted as a per cent of the control reciprocal. For comparison a similar curve for 2650 A ultraviolet is shown. It appears possible that the X-ray curve approaches a lower asymptote well above zero; *i.e.*, the maximum effect is a good deal less than complete cessation of division. The ultraviolet curve, on the other hand, appears to be asymptotic to zero or

Figure 1

Relation between dose and per cent of reduced vigor after autogamy for X rays from the Maxitron-250 machine. The points are weighted averages for all groups receiving approximately the same dose. Note that the dose scale is changed at the vertical line.

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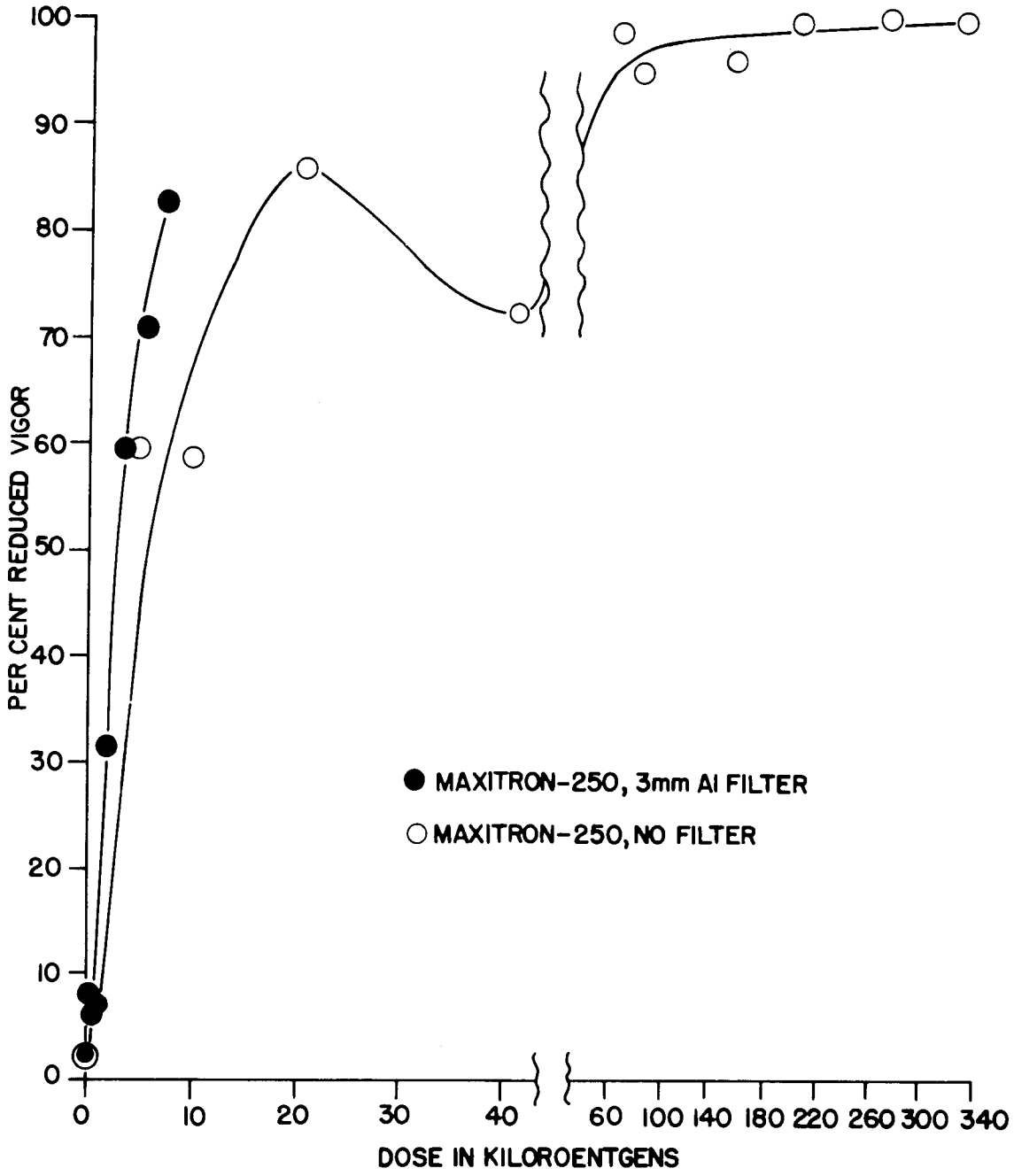
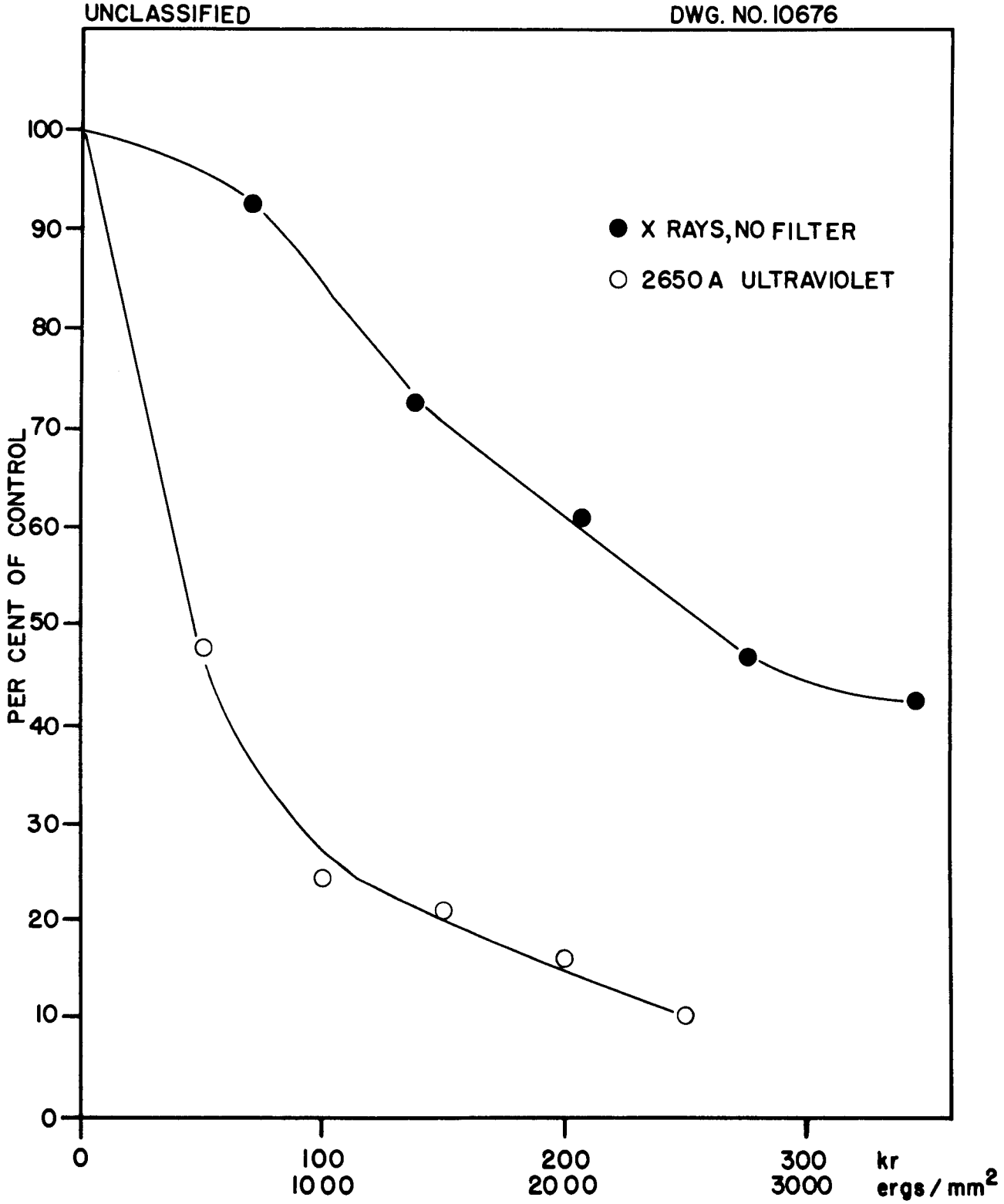


FIG. 1

Figure 2

Relation between dose of unfiltered X rays and reciprocals of the times to the sixth division expressed as a per cent of control. For comparison, previous results with 2650 A monochromatic ultraviolet are shown.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X RAYS, NO FILTER
2650 A ULTRAVIOLET
PER CENT OF CONTROL
100
90
80
70
60
50
40
30
20
10
0
0
100
1000
200
2000
300
3000
kr
ergs / mm²

FIG. 2

nearly so. This reflects the fact that in the ultraviolet material there is a long cessation of division after two to three divisions have occurred. With X rays such a cessation has not been demonstrated; and, if it occurs, it is certainly relatively less important than delays in the first division after irradiation. However, delays in divisions later than the first clearly occur in the X-irradiated material. Some delay is detectable after at least four and perhaps more divisions. Thus the delaying action is prolonged over several divisions just as with ultraviolet, but the quantitative relations between early and late effects apparently are not the same.

Yeast genetics. (Pomper, K. Daniels; Cheniae of Plant Physiology) Work is continuing on the *p*H-sensitive, complex mutant reported on in ORNL-889. The interpretation put forth in that report, *viz.*, that the P-aminobenzoic acid (PAB) requirement was the key genetic block, and that the mutant's additional requirements for adenine, histidine, and methionine (at *p*H 6.5) were caused by impaired PAB synthesis, still appears reasonable. Some clarification on the mutant's pantothenate (Pa) requirement is available. As noted in ORNL-889, the Pa requirement seemed also to be involved with the remainder of the complex system revolving about PAB, but the data were inconclusive. Experiments on reversion of the requirements (following ultraviolet irradiation) indicate that the Pa requirement is a separate gene (since two colonies picked from a plate lacking Pa were Pa-independent, but still required PAB, methionine, adenine, and histidine). However, colonies picked from other minimal plates (as, for example, from agar lacking adenine) which were now independent for all the factors of the complex, were also independent for Pa. The tentative interpretation that we are assigning these observations is that the need for Pa can be met by a reversal of the PAB requirement, but that the Pa requirement is probably due to a separate gene.

Contribution of genetic material and of phosphorus from the infecting parental phage to its progeny. (Doermann, Hill; with cooperation of Volkin of Biochemistry). The experiments undertaken in the previous quarter are preliminary to a more detailed investigation of the nature of the contribution of the infecting phage particle to its progeny. Genetically mixed infections have been made where one genetic type was uniformly labelled with P^{32}, the other unlabelled. The immediate purpose was to see whether a parallel exists in the progeny between the proportion of the radioactive genotype and the amount of P^{32} incorporation from that parent.

Bacteria were infected with five to ten (av.) particles of T4*r* as primary infection, and five to ten particles of the mutant T4*r* uniformly labelled with P^{32} as a secondary infection at intervals following the initial *r* infection. Phage reproduction was stopped and complete lysis induced by the addition of cyanide 10 minutes after the end of the normal latent period. It was necessary to separate the phage of the progeny from the medium and the bacterial debris in order to measure the radioactivity of the progeny. This was done by a series of differential centrifugations which was followed by differential centrifugation tests to decide whether the final phage was sedimented in parallel with the radioactivity. Small amounts of radioactive impurities could be detected in the low speed sediments and in the high speed supernatants. These totalled less than 20 per cent in every case. In the final phage obtained, the fraction of the original parental radioactivity was measured. The genetic contribution of the parent was also estimated by the proportion of *r* particles in the progeny. The results are shown in Table 2, columns II and III.

TABLE 2

Experiments with bacteria infected with nonradioactive T4r (primary infection) followed by radioactive T4r (secondary infection)

I DELAY OF SECONDARY INFECTION (min**)	II PER CENT *r* PHAGE IN PROGENY	III PER CENT OF PARENTAL RADIOACTIVITY IN PROGENY	IV PER CENT OF RADIOACTIVITY AS ACID SOLUBLE MATERIAL*	V PER CENT OF RADIOACTIVITY FOUND IN MEDIUM BEFORE LYSIS
0	65	32	9	14
2.5	20	17	27	38
5	5.4	13	32	48
10	1.7	8	31	42
16	0.6	7	31	47
20	0.6	11	32	39

*Measured 5 minutes after the secondary infection. The majority of this appeared in the medium.

**The latent period under conditions of these experiments is 30 minutes.

Evidently the ability of the infecting particle to contribute genetic material falls off more rapidly with time than its ability to contribute phosphorus. This may be interpreted to mean that the parent contributes appreciable nongenetic phosphorus which must be taken into account when one endeavors to link the genetic and the chemical aspects of phage reproduction. We are seeking more knowledge about these two types of phosphorus contribution by investigating more methods of differentiating them.

Recently Lesley, French, and Graham (Arch. Biochem., 28: 149-150, 1950) have discovered that in T2, a virus closely related to T4, a delayed secondary infection with radioactive phage results in the production of a large proportion of acid-soluble P^{32} compounds. Whereas the infecting particle contains less than 5 per cent of its phosphorus in acid-soluble form, the culture after delayed secondary infection contains 50 per cent of the P^{32} of the secondarily infecting type in acid-soluble constituents. Confirmation of this finding with T4, is seen in Table 2, columns IV and V. It was also found that the majority of this acid-soluble material is found in the culture medium rather than the cells, and that this medium always contains significantly more P^{32} than is accounted for by the acid soluble material.

EFFECTS OF RADIATION ON RATE OF MITOSIS

Mary Esther Gaulden (Leader)
J. Gordon Carlson*
W. K. Baker*
S. R. Tipton*
Elizabeth Sgourakis
Nyra Harrington
Marjorie Nix
R. W. Rogers**

Cytological effects of respiratory exciters and inhibitors and enzyme substrates. (Gaulden and Nix) This study has been initiated as basis for anticipated studies to determine the influence of these substances on radiation damage and for physiological studies of the grasshopper neuroblast.

Grasshopper embryos were dissected in isotonic culture medium and transferred to sterile dishes containing the desired concentration of the chemical. The embryos were then made into hanging-drop preparations and the neuroblasts observed under oil immersion lens of a microscope at 38° C for cytological effects and for mitotic inhibition.

*Consultant
**ORINS Fellow

70×10^{-5} *M*. Complete and immediate reduction in size of the spindle was observed in cells treated in prometaphase and metaphase with resulting failure of division.

10×10^{-5} *M*. Some cells observed in very late prophase and prometaphase immediately after immersion in dinitrophenol showed reduction in size of spindle at the subsequent metaphase. These cells do not go into anaphase. Some cells observed in metaphase at time of treatment also showed this reduced spindle. When cells were removed from dinitrophenol solutions, they divided normally. Cells treated in middle and late prophase reverted to early prophase stages. All stages of division were shown.

1×10^{-5} and $0.05 - 0.1 \times 10^{-5}$ *M*. Cells treated in very late prophase, prometaphase, and metaphase showed no cytological effect but cells treated in middle and late prophase showed reversion to early prophase stages.

$0.04 \times 10^{-5} - 0.3 \times 10^{-5}$ *M*. In some embryos neuroblasts treated in middle and late prophase reverted to earlier prophase stages but other embryos showed no cytological effect.

0.01×10^{-5} *M*. No cytological aberrations. In order to determine whether a cytologically ineffective concentration (0.01×10^{-5} *M*) of dinitrophenol causes retardation of mitosis, the mitotic rate of DNP-treated embryos was compared with that of control sister embryos. The mitotic rate was determined over a period of 6 hours as described in ORNL-727:41. At the end of the first 2 hours the embryos being treated with DNP were transferred to a hanging-drop of culture medium without dinitrophenol, and mitotic counts continued. The control embryos were transferred to hanging drops of fresh culture medium in order to duplicate the conditions of the treated embryos.

Statistical analysis by Mr. Jack Moshman of ORNL Mathematics Panel showed no significant difference between mitotic rate of embryos in 0.01×10^{-5} *M* dinitrophenol and that of sister control embryos.

A seasonal slump in available eggs has limited further work to preliminary experiments with sodium azide and sodium formate.

SODIUM AZIDE

0.001 *M.* Reduction in size of spindle was observed in cells treated in prometaphase and metaphase. Cells treated in very late prophase advanced to metaphase but the spindle was small and in all three cases further division did not occur. Cells treated in middle and late prophase reverted to earlier prophase stages.

0.0001 *M.* No cytological aberrations.

SODIUM FORMATE

0.05 *M.* Cells treated in middle and late prophase reverted to early prophase stages.

0.03 *M.* Some cells treated in middle and late prophase reverted to early prophase stages. Others were unaffected.

0.01 *M.* No cytological aberrations.

Effect of X radiation on desoxyribonucleic acid (DNA). (Harrington; Koza of Biophysics) A paper entitled "Effect of X Radiation on the Desoxyribonucleic Acid and on the Size of Grasshopper Embryonic Nuclei" has been completed. Cytological and cytochemical studies were made on embryonic nuclei of the grasshopper, *Chortophaga viridifasciata,* after X-ray doses of 4000, 10,000, and 12,500 r.

Photometric measurements were made, using the Feulgen reaction, to determine relative changes in the DNA pentose and the methyl green stain to indicate the degree of polymerization of the nucleic acid. Statistical treatment of the Feulgen-stained nuclei showed no significant loss of DNA after irradiation, but the data on nuclei stained with methyl green disclosed a highly significant loss of stainability after X irradiation. Depolymerization of the nucleic acid appears to have taken place. The visible loss of Feulgen-stainability is believed to be due to the enlarged nuclei and to the physical change in the nucleic acid.

Observations on living tissue culture preparations indicate that an immediate swelling of the nuclei occurs after irradiation. The change in the

tonicity of the cells may be a result of the depolymerization of DNA. Measurements of stained nuclei reveal a significant increase in the nuclear diameters after radiation. The degree of swelling is not proportional to the dose of X radiation given.

Immediate effects of X radiation on mitotic stages. (Carlson, Harrington) Living culture preparations of grasshopper neuroblasts are being observed under the microscope and then given an X-ray dose of 64, 256, 1024, or 4096 r within the space of 1 minute. A very detailed and accurate study is being carried on in which the effects of irradiation on each stage of mitosis from late prophase to anaphase are noted. Such effects as the appearance of chromosome stickiness, the degree of mitotic retardation, and cleavage delay are observed. The experiment is still in progress, but enough data have been recorded to show that, though the cleavage furrow may be delayed for several hours by the extreme stickiness of chromatin, cytoplasmic division does eventually occur. Cleavage is normally complete when the chromosomes have separated in late anaphase, but after extreme doses of X rays, the chromatin may have the appearance of a middle telophase without the cytoplasmic division. In nearly every experiment the furrow is complete before the nucleolus has reappeared.

EFFECTS OF RADIATION ON MICROORGANISMS

A. Hollaender (Leader)
G. E. Stapleton
R. B. Grayson
W. T. Burnett, Jr.*
M. L. Morse**
Arthur Burke**

Modification of X-ray damage by chemical compounds and oxygen. The survival of bacteria exposed to X radiation has been shown to be strikingly increased by a reduction of the oxygen tension of the bacterial suspension prior to irradiation. Cysteine or glutamic acid added before irradiation has also been shown to modify the X-ray sensitivity of bacterial cells. An investigation is now in progress to find additional compounds which will materially reduce the degree of damage produced in living cells by ionizing radiations.

As in previous experiments, the survey for new protective agents is made using washed cell suspensions of *Escherichia coli* (B/r) cultured 18-20 hours

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in nutrient broth (Difco). The cells are washed and concentrated in 15 *M* phosphate buffer by centrifugation, the final volume of buffer being about one-tenth the original volume. An aliquot of the suspension containing about 10^9 cells/ml is introduced into a 2-ml test tube together with about 1 ml of a solution of the compound to be tested and exposed, in equilibrium with air, at ice bath temperature, unless otherwise specified. The source of X rays is a Maxitron-250 unit operated at 250 kvp at 30 ma (3 mm added aluminum filter). The dose at about 25 cm is 1000 r/min and at 17 cm, 2000 r/min. The irradiated suspension is diluted and aliquots transferred to agar plates, the number of colonies developing in 24 hours at 37° C being considered to represent the number of surviving cells.

The general scheme of the survey is as follows:

ROUGH SCREENING

The cells are irradiated at a constant dose (60 kr) in a solution of the compound (0.02 *M*) buffered to *p*H 6.8. The following three types of control are used.

1. An unirradiated suspension in phosphate buffer to test the toxicity of the compound under our conditions.

2. An irradiated suspension in buffer to determine the number of survivors without benefit of a protective agent.

3. An irradiated suspension in buffer containing cysteine (0.02 *M*) which, for our purposes, is being used as a standard protective agent.

This general procedure was considered plausible since it was initially thought that any compound, to be of further interest, should give at least the same order of magnitude of protection as cysteine.

Compounds which have appeared promising by the screening procedure have been further characterized by the following investigations.

1. Survival fraction as a function of X-ray dose in the presence and absence of the compound.

2. Survival as a function of concentration of the compound as a constant X-ray dose, which yields information as to the degree of protection as well as the efficiency of the protective compound.

3. Survival as a function of dose under high and low oxygen concentrations. This portion of the study should give information as to whether or not the protection afforded by the compound is related to the oxygen concentration effect.

4. Additivity of different protective systems or agents.

RESULTS

This portion of the study indicated that many compounds are able to afford some degree of protection; however, relatively few to date appear sufficiently promising to warrant intensive characterization. The compounds found to be protective seem to fall into several classes on the basis of their chemical structures as well as of their metabolic importance. A crude and subjective classification is as follows.

1. Compounds containing sulfhydryl groups: cysteine, mercaptosuccinate, 2,3-dimercaptopropanol (BAL), and 2-(2-mercaptoethoxy) ethanol.

2. Alcohols and glycols (under restricted conditions): methanol, ethanol, propanol-2, propanediol, glycerol, triethylene glycol, and propylene glycol.

3. Redox compounds: sodium dithionite.

4. Metabolic intermediates and products (under restricted conditions): formate, succinate, pyruvate, fumarate, lactate, and malate.

CHARACTERIZATION — RESULTS

The characterization of the protective agents is still in the preliminary stages. However, typical data for the portions of the characterization are given in graphic form in Figs. 3 and 4 for the sulfhydryl compounds, cysteine and BAL. Figure 3 shows the survival fraction of the bacterial cells as a function of X-ray dose in the presence and absence of the sulfhydryl compounds. It is evident that BAL is about twice as effective as cysteine on the basis of the relative dose required to inactivate the same fraction of cells in oxygen-saturated suspension. Moreover, cysteine is as protective in nitrogen-saturated as in oxygenated suspensions, possibly indicating that the sulfhydryl

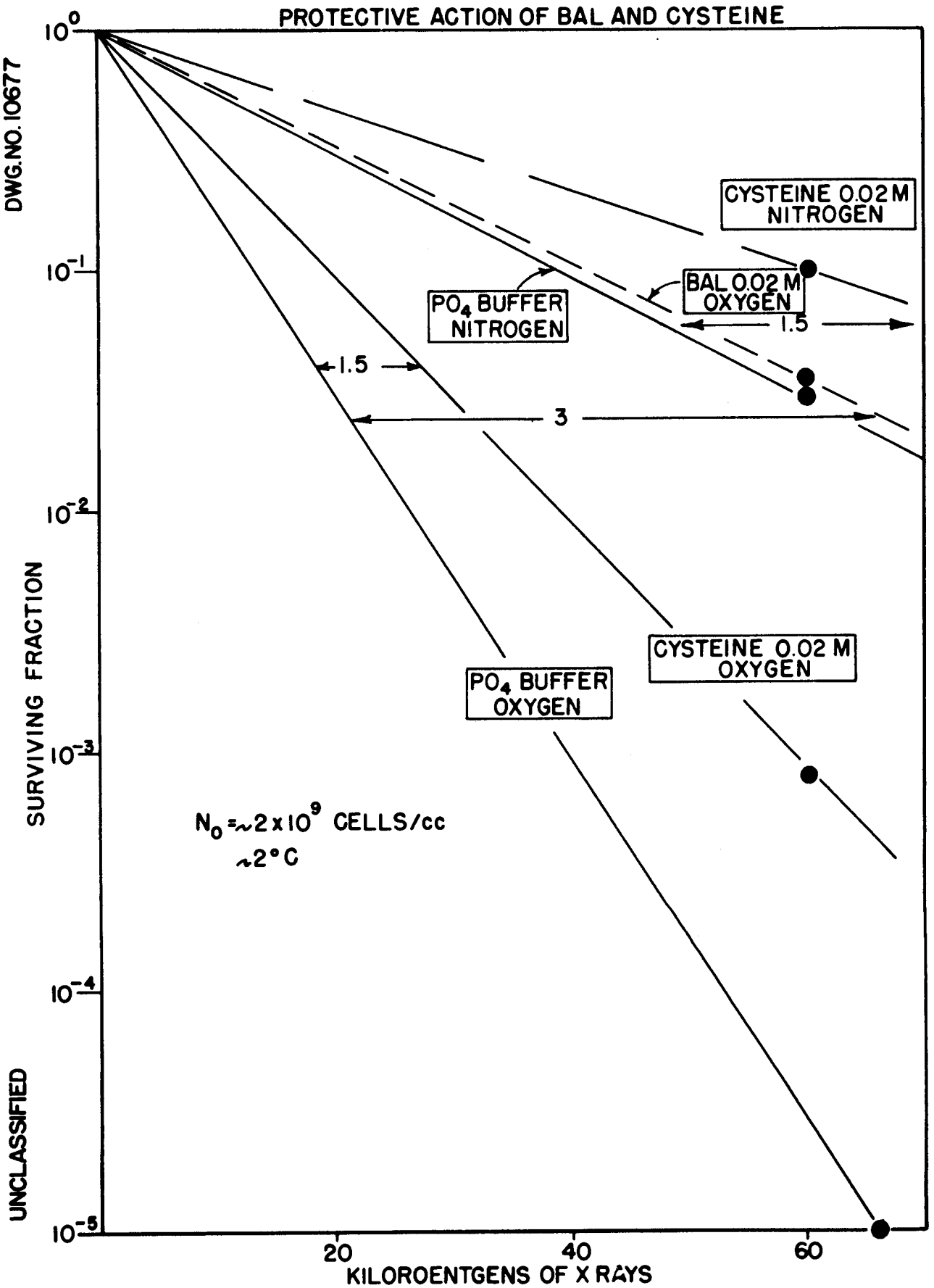

FIG. 3

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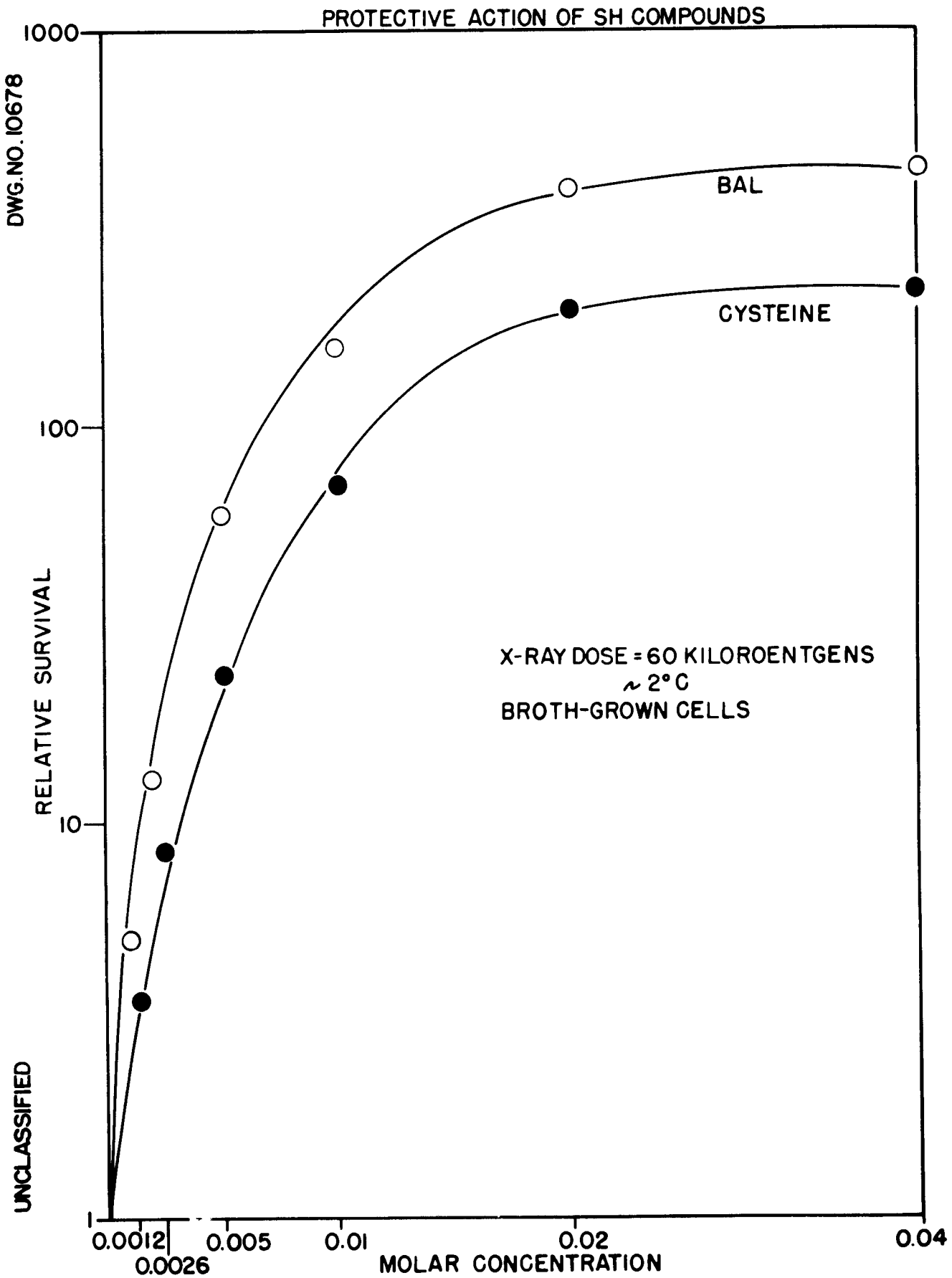

FIG.4

UNCLASSIFIED

groups protect by a mechanism different from removal of oxygen from the irradiation medium alone. Figure 4 is a plot of relative survival as a function of molar concentration of the two compounds, at a constant dose of X rays, with suspensions in equilibrium with atmospheric air. The differences in the slopes of the curves for the two compounds again indicate the twofold efficiency of BAL over cysteine. These curves are of interest since the optimum or limiting concentration for both sulfhydryl compounds is about 0.02 *M*. Other experiments have shown that addition of increasing concentrations of cysteine to the optimum concentration of BAL failed to increase the survival, whereas below the optimum concentration of either compound, the compounds are completely additive in their protective capacity. The greater survival at optimum concentration for BAL might be explained by the fact that the parent compound of BAL, propanol, is also somewhat protective.

Doermann found that there is a linear relation between protective capacity and concentration of glycerol in the higher concentration ranges. More complete investigation of the glycols as well as more simple alcohols has revealed that all of them have essentially similar characteristics both in regard to the concentrations required to give detectable protection as well as the degree of protection afforded by optimum concentrations. The concentrations required to give protection are of the order of a hundred times that required for the sulfhydryl compounds, as shown in Fig. 5. It should be pointed out that some of the higher concentrations of alcohols used here are toxic under conditions other than those used in these experiments in which the temperature was always held near 0° C.

It is of interest that sodium dithionite, $Na_2S_2O_4$, a strong reducing compound, gives protection in exceedingly low concentrations. Moreover, dithionite protects in nitrogen-saturated suspensions. This compound appears to protect either by further depletion of oxygen from nitrogen-saturated suspensions, or else its protection is independent of the oxygen concentration. As can be seen from Fig. 6, 10^{-4} *M* dithionite is protective.

Some of the carboxylic acids which are normal metabolic intermediates or end products have also proved to afford excellent protection to bacterial cells, but again under rather restricted conditions. Low protective capacity can be demonstrated for all the acids mentioned under our standard conditions;

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PROTECTIVE ACTION OF ALCOHOLS

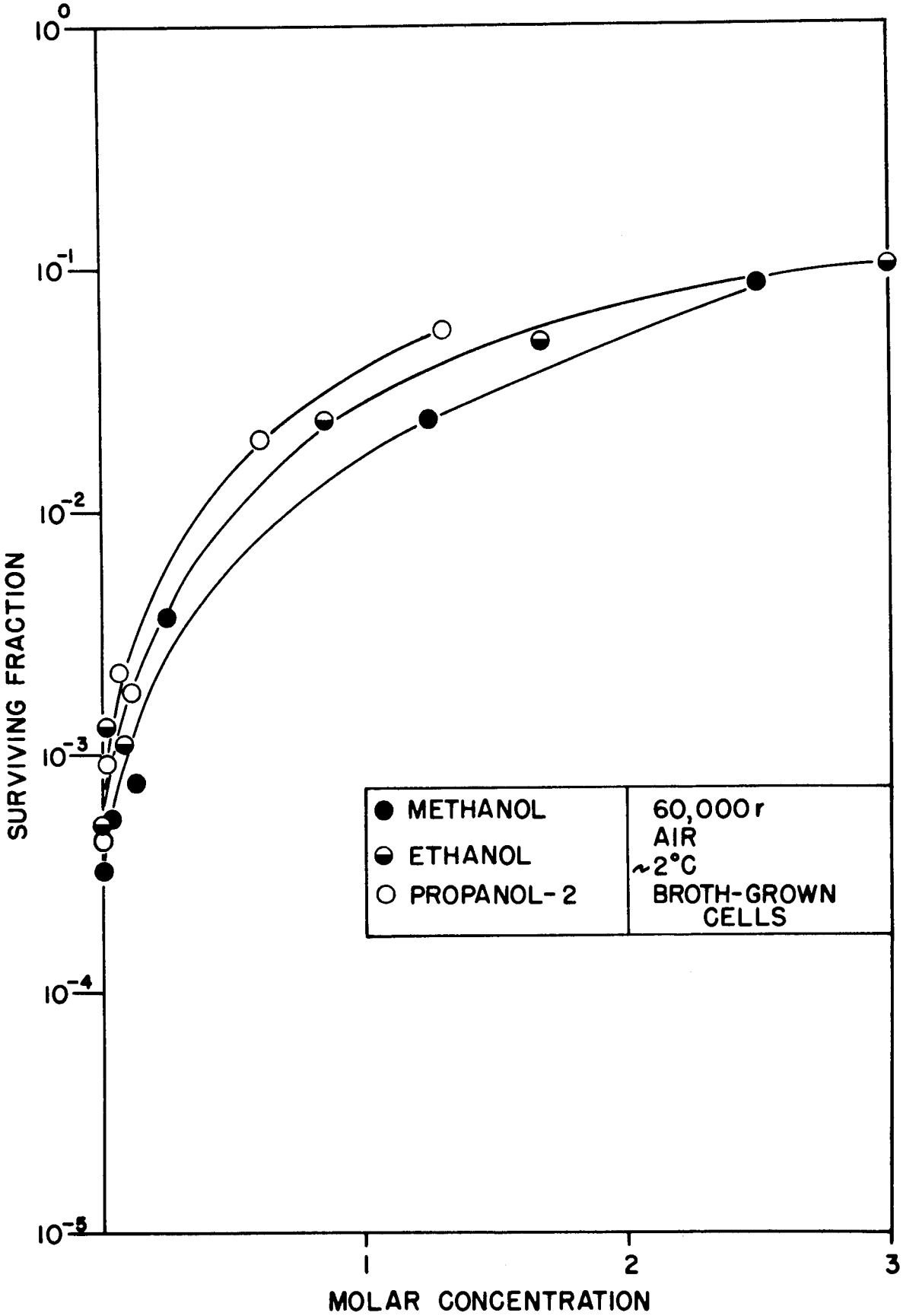

FIG. 5

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DWG.NO. 10680

PROTECTIVE ACTION OF BAL AND SODIUM DITHIONITE

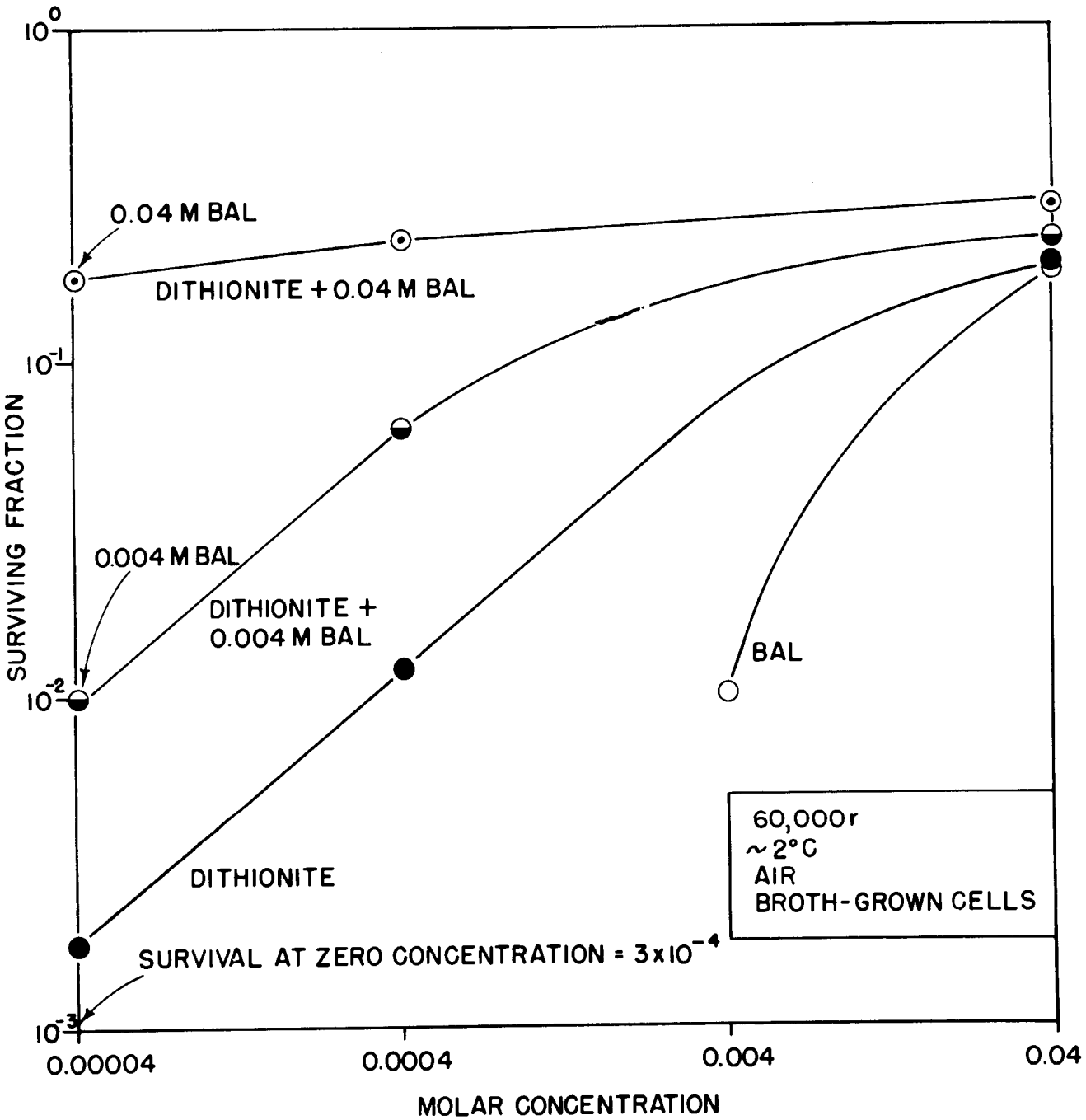

FIG.6

however, allowing a short incubation period prior to irradiation for the cells in the presence of the acids has proved to increase the protection. The probable mechanism is depletion of oxygen from the suspension by the bacterial cells in the presence of a utilizable substrate. The ability of BAL, cysteine, dithionite, and metabolic intermediates to protect bacterial cells appears to warrant further investigation.

It is of particular interest that, when two or more types of protective agents were used in combination, the effect was additive (Fig. 6). For example, when BAL was combined with an alcohol, dithionite, cysteine, or formate, additional protection was afforded. Such additivity suggests that there are several mechanisms for protection.

A program using small laboratory animals to test potential protective agents has been set up in cooperation with the University of Chicago (A. M. Brues and H. M. Patt). A number of compounds screened by the bacteria method have already been turned over to this group for further investigation; a few are being tested on a small scale in our laboratory, using mice. This phase of the program will be treated in subsequent reports.

Valuable suggestions for compounds to be tested have been made by Dr. C. T. Bahner, Carson-Newman College; Mr. L. E. Klein, Monsanto Chemical Company; and Dr. D. G. Doherty, Biology Division.

The effect of oxygen on the X-ray induction of mutations in bacteria. (Anderson, Whittle). This study has been extended to include the oxygen effect on the X-ray induction of the back mutation or reversion to proline independence of a prolineless radiation-resistant mutant of *Escherichia coli* (B). The results obtained with this mutant show that oxygen has a profound influence in increasing the back mutation rate to proline independence. The dose-reduction value of oxygen for the lethal effect of X rays for this mutant was found to be 3.0 while the dose-reduction value of oxygen for the genetic effect was found to be approximately 2.5. This may be compared with a dose-reduction value of oxygen of 3.0 for both the lethal and genetic effects of X rays earlier reported for a purineless mutant (ORNL-807). Thus it is seen that although the presence of oxygen increases the efficiency of X rays to the same degree in producing lethal effects in these two biochemical mutants, it does not increase the efficiency of X rays to the same degree in producing the

two genetic effects studied. The information obtained with these two mutants and that with a streptomycin-dependent mutant, which showed but a very slight influence of oxygen on the X-ray-induced back mutation to streptomycin nondependence (ORNL-807), indicates that oxygen may show a complete spectrum of magnitude in increasing the efficiency of X rays in producing different genetic effects in *E. coli*.

MAMMALIAN GENETICS AND DEVELOPMENT

GENETIC AND DEVELOPMENTAL EFFECTS OF RADIATION IN MICE

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Josephine S. Gower
J. C. Kile, Jr.
Louis Wickham
Jane E. Crowell
Mary Henderson
Gloria J. Jones
Elizabeth M. Kelly
Patricia A. Sarvella

Determination of mutation rates at certain chosen loci. (W. L. Russell, Gower, Henderson, Jones) The method has been outlined in earlier progress reports and consists essentially of mating irradiated wild-type mice, and nonirradiated controls to a strain homozygous for as many known recessive genes as can be managed practically (seven in the present experiment). A mutation at any of the loci represented by the recessives will be detected in the first generation.

Some of the information obtained from a pilot experiment has already been presented (ORNL-807). Enough data have now been accumulated in the main experiment to warrant a preliminary estimate on the mean induced mutation rate of the seven loci tested. The results in the experimental group reported here were obtained from males exposed to a single whole-body dose of 600 r of 250 kvp X rays at an intensity of approximately 90 r per minute. Matings were made after the exposed animals had recovered from their temporary sterility, the presterile period not being used in this particular experiment. Thus the male germ cells that produced the F_1 offspring had been exposed in spermatogonial stages. The nonirradiated group is mostly from wild-type males x test stock females, but includes some data from the reciprocal cross.

The mutations obtained to date are given in Table 3. They have been

TABLE 3

Presumed mutations at specific loci

	NO. OF F_1 ANIMALS	NUMBER OF MUTANTS LOCUS							TOTAL
		A	*B*	*C*	*D*	*P*	*S*	*Se*	
Irradiated (600 r)	22,610	-	6	1	2	5	12	-	26
Nonirradiated	27,729	-	-	-	1	-	-	-	1

called "presumed" mutations because the evidence that the specific loci are involved and that they are "gene," as opposed to chromosomal, changes is not yet complete. The former point has, however, been established for the first four mutants on which genetic tests are complete. Some of the other mutants have been partially tested. Furthermore, the phenotype, except in the case of spotting (*S* locus), is, if carefully examined, good evidence in itself. There is, so far, no evidence that the effects are due to chromosomal changes, rather than point mutations, except possibly in some of the spotted animals. The evidence is far from complete on this question, but from earlier work of others on irradiated spermatogonia, it appears unlikely that gross chromosomal changes can be involved.

Making a conservative assumption by assigning to the *S* locus a value that is the mean of the remaining six loci, gives an induced (experimental minus control) rate of $(1.63 \pm 0.43) \times 10^{-7}$ per locus per roentgen. Assuming that none of the mutations is spurious gives an upper value of $(2.65 \pm 0.54) \times 10^{-7}$. Further testing should eliminate most of the guess-work concerning the actual number of true mutations, but at the present time it can be stated with considerable confidence that the true value for the mean induced mutation rate per roentgen of the seven loci tested lies between two standard errors below the lower value given above and two standard errors above the upper value, *i.e.*, between 0.77×10^{-7} and 3.73×10^{-7}.

Current estimates of the genetic hazards of radiation in man are based largely on radiation-induced mutation rates in *Drosophila*. Since the present rates in the mouse are based on a limited number, and not a random sample, of loci and on one type of mutation (recessive visible), the most meaningful use to which they can be put, with regard to human hazards, is to compare them with parallel data in *Drosophila*. Muller, in an experiment essentially similar to the one described here, has for some time been collecting data on fifteen specific loci in *Drosophila*. Preliminary reports of this work indicate that the mean radiation-induced rate per roentgen of these fifteen loci is, for irradiated gonia, probably not higher than 1.5×10^{-8}. The lower and upper values obtained above for the mouse, which allow both for sampling errors and for some uncertainty as to the validity of classification of the mutants, are respectively five and twenty-five times the *Drosophila* figures.

Although this is only a preliminary report, it is already clear that the method should yield mutation rate estimates of a far greater reliability than

we had expected. They seem reliable enough, with the data obtained so far, to justify the statement that the radiation-induced mutation rate in one mammal is considerably higher than that in *Drosophila* for at least one type of mutation in small, but similarly selected, samples of loci. If we wish to generalize from this, and there is little choice at the present time, the data point a warning that estimates of human hazards based on *Drosophila* mutation rates may be too low by approximately one order of magnitude.

PATHOLOGY AND PHYSIOLOGY

Jacob Furth (Leader)
M. C. Woods — J. J. Lane
J. B. Kahn — W. D. Gude
R. H. Storey — Peggy Ledford
Mary H. Ross — Germaine Click
R. R. Bigelow* — Mary M. Knoohuizen
K. W. Christenberry*

Chronic changes by slow neutrons and equivalent doses of X rays. (Ophthalmology, Christenberry; assistance, Lane, Gude, Ledford) The setup of this experiment outlined in an earlier report is complete. Early changes have already been described. The animals are now followed until natural death.

The most common neoplasms induced thus far (6 to 16 months after exposure) by both X rays and thermal neutrons are leukemias, and the most prevalent nonneoplastic change is cataract. Table 4 confirms, in general, earlier progress reports on the leukemia induction rates in this series. The per cent of

TABLE 4

Progress survey of leukemia incidence in mice exposed to slow neutrons (Made 6 - 16 months after irradiation)

	THERMAL NEUTRONS (Minutes Exposure)				X RAYS (Minutes Exposure)			CONTROLS
	80*	20-40	5-10	2.5	80	20-40	5-10	
No. in group	243	261	275	73	159	161	214	289
Per cent leukemia	15.2	10.7	2.2	2.7	17.6	10.5	2.8	2.8

* Thermal neutron flux of 0.9×10^9 n/cm^2/sec. with high energy contaminant of about 4-6r/min.

250 kvp; 12.5 ma; 3.5 mm Cu; TSD 93.7 cm; rate 6.4r/min.

leukemias in the controls has increased; with increasing age many more of the control animals develop leukemias, while the radiation-induced neoplasms occur at an earlier age. Doses of 128 r and equivalent doses of neutrons increased the leukemia incidence about fourfold; 512 r and equivalent neutrons, about

* Consultant.

sixfold. A preferential effect of neutrons as shown for cataract induction is not evident for leukemia induction.

Neoplasms other than leukemia now appear in the series, *e.g.*, lung, ovarian, soft tissue, and bone tumors, but the data are too meager for analysis.

The cataract induction data are essentially the same as reported earlier. The 32 r (5-minute series) and equivalent neutrons did not increase the leukemia incidence but induced nuclear opacities which are detectable as early as 4 to 6 months after after irradiation.

Since the γ rays of the pile flux are of high energy, it seemed desirable to determine the LD-50 of similar gammas in comparison with LD-50 of X rays for mice used in this series. The cobalt gamma was used for this purpose. The LD-50 was found to be about 900 r as compared to about 620 r for 250 kv X rays as employed.

ACUTE RADIATION DAMAGE

I. *Permeability problems.* (Surgery, Bigelow; chemistry, Woods, Storey; assistance Knoohuizen, Ross) Lymph studies have confirmed the conclusion reached in earlier work, namely, that the radiation anemia is due in part to diversion of red blood cells into the lymph compartment (Furth, Andrews, Storey, and Wish, Sou. Med. J., 44:85, 1951).

Tables 5 and 6 present summaries of recent experiments illustrating this hitherto neglected basic event in the hemorrhagic syndrome.

TABLE 5

White and red cell counts in lymph and blood of normal and irradiated dogs

	LYMPH		BLOOD	
	W.B.C. x 10^3	R.B.C x 10^3	W.B.C. x 10^3	R.B.C. x 10^6
Normal (4)				
Extremes	26.5-39.7	0.4-7.8	19.4-23.3	5.0-6.6
Average	32.4	3.2	20.7	5.8
Dog I				
Days post 350 r				
8	0.5	125.0	1.0	5.3
10	1.0	115.0	1.0	3.5
13	0.4	155.0	0.3	5.1
17	0.4	240.0	0.4	4.3
Dog II				
Days post 350 r				
2	3.8	3.3	4.5	-
6	4.4	2.1	2.1	-
8	1.0	134.5	2.0	-
11	-	998.5	2.0	-
13	3.0	1060.0	0.3	-

TABLE 6

White and red cell counts in lymph and blood of normal and irradiated rats

	LYMPH		BLOOD	
	W.B.C. x 10^3	R.B.C. x 10^3	W.B.C. x 10^3	R.B.C. x 10^6
Normal (5)				
Extremes	5.4-22.5	0-1.5	12.5-21.3	8.0-10.2
Average	15.0	0.31	14.3	9.0
X-rayed* (8)				
Extremes	0.2-1.5	23.4-1070.0	0.3-3.2	4.6-10.0
Average	0.7	523.2	1.0	7.6

* 6 to 11 days after 650-750 r

Analyses of the protein concentration of blood and lymph by chemical and electrophoretic methods in both normal and irradiated dogs are in progress. Lymph clearance studies of large molecular tagged substances introduced into circulation are being continued.

II. *Disappearance rate of tagged substances from the blood.* (Cooperative work with Radiobiochemistry group) The study on the disappearance rates of tagged substances from the circulation of X-rayed and normal animals has been completed and the paper describing it ("Disappearance Rate of Tagged Substances from the Circulation of X-rayed Animals" by Wish, Furth, Sheppard and Storey) has been submitted for publication. The disappearance of I^{131}-labeled homologous and heterologous plasma, P^{32}-labeled homologous and heterologous erythrocytes, Evans Blue, and colloidal Au^{198} was studied in rabbits and mice. All these substances were found to disappear faster from the circulation of X-rayed than from normal animals. Table 7 summarizes ratios of disappearance rates in X-rayed/normal rabbits and is indicative of the trend observed.

These observations have been interpreted in the light of the lymph fistula studies, which they preceded, as indicating an increased capillary permeability after irradiation.

It was found that phagocytic activity by macrophages is enhanced in X-rayed animals because of greater demand, but phagocytic ability appears not

TABLE 7

DAYS AFTER IRRADIATION	HOMOLOGOUS PLASMA	HETEROLOGOUS PLASMA	HOMOLOGOUS CELLS	EVANS BLUE
1	1.0	0.9	1.9	0.8
2-5	2.3	1.2	2.4	1.2
6-9	3.1	1.5	3.2	1.4
10-13	2.8	2.9	2.4	
14-17			3.9	1.3
21	1.8			

significantly altered. The extraordinary rapidity of phagocytosis of intravenously injected particles was demonstrated by radioisotope methods and related to liver circulation. The rapid breakup of foreign erythrocytes within reticulo-endothelial cells was quantitated by measurement of the loss of P^{32} activity used for tagging.

Pathogenesis of radiation anemia. (Kahn, Storey; assistance, Click) The basic design of this experiment has been stated in our last report (ORNL-889): rabbits are given intravenous injections of equal quantities of Fe^{59}-tagged erythrocytes. Some are irradiated immediately before and some immediately after this injection. The controls are not irradiated.

An additional carefully planned experiment of this type was performed in which both plasma and cell volumes were determined at various times after the injection of Fe^{59}-tagged erythrocytes. White-cell counts and reticulocyte counts were made periodically to ascertain the status of bone marrow activity. These results confirm earlier experiments indicating that 800 r does not injure erythrocytes *in vivo*, but irradiation of the hosts with this dose results in a drop of the erythrocyte mass which in some animals is far greater than can be accounted for by death of aging erythrocytes. Details of this experiment will be reported later.

A discrepancy was noted between erythrocyte mass as determined with Fe^{59}-tagged cells on the one hand and P^{32}-tagged cells on the other hand. The cause of this discrepancy remains to be determined. The erythrocyte mass as determined by Fe^{59} tagging is significantly lower than that indicated by the

P^{32} technique. If the Fe^{59} technique is correct, as we believe it to be, the conversion value from peripheral hematocrit to average body hematocrit is 0.83. If the radiophosphorus technique is correct, this conversion value is 0.87.

Long-term effects of C^{14}. (Woods, Storey) The construction of quarters for the care of animals exposed to radioisotopes, and laboratory facilities for the use of C^{14}-labeled compounds in metabolism studies, have been completed. Orientation experiments were set up using glycine-2-C^{14} to permit high-level incorporation and retention of C^{14}. The general and special control animals are maintained under comparable conditions. Urine, feces, and expired carbon dioxide are being analyzed for C^{14}. The animals treated thus far number 51 and the controls 46.

MICROBIOLOGY

TRACER STUDIES ON METABOLISM

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E. H. Mosbach Mary V. Long
E. F. Phares Betty Ann Gwin
F. W. Denison, Jr.*

Degradation of α-ketoglutaric, glutamic, and citric acids. The work on a new, stepwise degradation procedure for α-ketoglutaric acid (outlined in the preceding report, ORNL-889, p. 44) has been completed. It was found that the method could be extended to include other acids of biological importance, *i.e.*, citric, glutamic, and butyric acids. The method in its final form was modified in several very important respects, both to improve the yields (Steps 1 and 2) and also to eliminate a possible source of error (Step 3).

The degradation method was tested with α-ketoglutaric acid-1,2-C^{14}, which has been synthesized essentially as described by Kögl *et al.*, (Rec. Trav. Chim., 68:387, 1949). The results (presented below) indicated that the method resulted in a minimum of cross-contamination. The conversion yields obtained for the intermediates were such that the entire degradation procedure could be carried out with the following amounts of total radioactivity: citric acid, 0.5 μc; α-ketoglutaric acid, 0.2 μc; glutamic acid, 0.1 μc; butyric acid, 0.05 μc. These figures apply to Geiger-Müller counting under the conditions used in this laboratory.

1. CONVERSION OF CITRIC ACID TO α-KETOGLUTARIC ACID

(Carson, Mosbach, Gwin)

The known biological dissymmetry of citric acid (the Ogston Concept) made it necessary to degrade citric acid to α-ketoglutaric acid by an enzymatic method in order to preserve the distinction between the primary carboxyls of citric acid. The conversion was carried out with a pigeon breast-muscle preparation according to Krebs and Johnson (Enzymologia, 4:148, 1937), except that α-ketoglutarate was isolated by partition chromatography and not as the 2,4-dinitrophenylhydrazone. Preliminary studies indicated that the conversion of citrate to ketoglutarate amounted to 40 to 60 per cent of the theoretical.

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2. REDUCTIVE AMINATION OF α-KETOGLUTARIC ACID TO DL-GLUTAMIC ACID

(Mosbach, Long)

Step 1 as reported in ORNL-889 is followed in this experiment. It was found that the use of a 5 per cent palladium chloride-on-carbon catalyst (Org. Syntheses, 26:77, 1946) resulted in more reproducible yields. A typical reductive amination was carried out in the following manner:

> Into the 500-ml reduction bottle of the Parr hydrogenation apparatus were placed 500 mg of catalyst and 10 ml of water. The catalyst was reduced for 2 to 4 hours under a hydrogen pressure of 2.5 atmospheres. At the end of this period approximately 3 m*M* disodium α-ketoglutarate were added in 6 ml of water. The calculated amount of 30 per cent ammonia solution was then added to make the final ammonia concentration of the solution 4 per cent. Preliminary studies had shown that final ammonia concentrations of 2 to 5 per cent gave optimum yields of glutamic acid without undue prolongation of the reduction period (16 to 24 hours). The amination of the keto acid was carried out under a hydrogen pressure of 2 atmospheres for a period of approximately 16 hours. At the end of this period the catalyst was filtered off, and the solution was evaporated to dryness under reduced pressure in order to evaporate most of the ammonia. (The evaporation step was found to be necessary because the presence of excess ammonia resulted in high carbon yields in the subsequent ninhydrin reaction.) The residue was taken up in 20.0 ml of water and a convenient aliquot (0.2 m*M*) was taken for the ninhydrin reaction.

3. CONVERSION OF FLUTAMIC ACID TO SUCCINIC ACID SEMIALDEHYDE (Step 2)

(Mosbach)

In order to obtain higher yields, the oxidation of glutamic acid to succinic acid semialdehyde was carried out by means of the chloramine T reaction (Dakin, Biochem. J., 11:79, 1917):

> The aqueous glutamate solution from step 1 (18 ml) was placed into a 50-ml flask and stirred with 97.5 per cent of the theoretical quantity of chloramine T. (An excess of reagent must be avoided in order not to overoxidize glutamic acid to cyanopropionic acid). An inert gas (nitrogen or helium) was bubbled through the solution for 5 minutes at room temperature and the flask was then immersed into a constant-temperature bath at 50° C. The nitrogen flow through the solution was maintained until the reaction was complete

as indicated by a negative starch-KI test. The reaction mixture was cooled in an ice bath to 5° C and the precipitate of P-toluenesulfonamide was filtered off and discarded. Yields of this step were not determined, but succinic acid semialdehyde was identified as the P-nitrophenylhydrazone, M.P. 178-180° C and as the 2,4-dinitrophenylhydrazone, M.P. 198-200° C.

4. WOLFF-KISHNER REDUCTION OF SUCCINIC ACID SEMIALDEHYDE. (Step 3)

(Mosbach)

The directions were followed exactly as reported previously, with the very important exception that diethylene glycol was substituted for triethylene glycol. This change was necessary because it was found that triethylene glycol decomposed under the conditions of the experiment yielding appreciable amounts of butyric and propionic acids as well as considerable amounts of acetic acid. The butyric acid was produced in sufficient quantity to produce a 20 to 30 per cent dilution of the radioactivity of the labeled butyric acid. On the other hand, diethylene glycol yielded only small amounts of acetic acid under identical conditions, and it was quite feasible to separate butyric and acetic acids by partition chromatography.

5. SCHMIDT DEGRADATIONS OF BUTYRIC, PROPIONIC AND ACETIC ACIDS

(Phares and Long)

These were carried out as described previously. Table 8 summarizes the yields obtained in two typical degradations of labeled α-ketoglutaric acid.

TABLE 8

Conversion yields in the degradation of α-ketoglutaric acid

REACTION	PER CENT YIELD EXPT. 1	PER CENT YIELD EXPT. 2
α-ketoglutaric acid ⟶ glutamic acid	62.3	60.2
Glutamic acid ⟶ butyric acid	40.5	52.0
Butyric acid ⟶ propionic acid	74.6	75.8
Propionic acid ⟶ acetic acid	69.8	65.9
Acetic acid ⟶ methylamine ⟶ CO_2	69.8	69.5

Table 9 shows the data obtained in two degradations of α-ketoglutaric acid-1,2-C^{14}. The data indicate that within the estimated over-all precision of measurement (± 5 per cent) there is good agreement among the calculated and found values of the various carbon atoms examined.

TABLE 9

Degradation of alpha-ketoglutaric acid-1,2-C^{14}

$(\underset{1}{HOOC^{14}}-\underset{2}{C^{14}}O-\underset{3}{CH_2}-\underset{4}{CH_2}-\underset{5}{COOH})$

COMPOUNDS OR CARBONS	SPECIFIC RADIOACTIVITY: COUNTS PER MIN., PER MG. $BaCO_3$			
	EXPERIMENT 1		EXPERIMENT 2	
	CALCULATED	FOUND	CALCULATED	FOUND
α-Ketoglutaric acid (C-C-C-C-C) 1 2 3 4 5	-	7.50	-	9.48
C-1	18.9	18.1	23.6	22.6
Butyric acid (C-C-C-C) 2 3 4 5	4.73	4.72	5.94	5.99
C-5	0.00	0.048	0.00	0.072
Propionic acid (C-C-C) 2 3 4	6.50	6.12	7.92	7.50
C-4	0.00	0.036	0.00	0.072
Acetic acid (C-C) 2 3	9.50	9.24	11.8	11.3
C-3	0.00	0.24	0.00	0.40
C-2	18.9	18.1	23.7	23.4

The degradation procedure therefore permits the measurement of the isotope concentration of each separate carbon atom of citric, α-ketoglutaric, glutamic, and butyric acids. This method should prove useful in investigations of the biosynthetic mechanisms involved in producing these or related acids both in microorganisms and animal tissues.

Carboxy-hydrogen reaction. (Carson, Gwin) *Clostridium aceticum* allegedly carries out a biosynthesis of acetate from carbon dioxide plus hydrogen. We have reported that a satisfactory growth medium had been devised which would be suitable for preparation of resting cells—for use in

tracer experiments (ORNL-889:48). The completely sealed tubes (rigorous exclusion of oxygen) were only successful for growth of relatively small numbers of cells, and these were never found to be in an active physiological condition. Means have now been found whereby 1-liter quantities of medium can be used to prepare a heavy crop of physiologically active cells in 3 to 4 days. Sterilized platinum, glass, and calomel electrodes in the culture flask allow continuous check on the *p*H, which has been found to be optimum at 8.0 to 8.5, as well as the Eh, which must be maintained below -300 mv (optimum -350 to -400 mv). The *p*H can be maintained with appropriate mixtures of carbon dioxide and hydrogen; the gas mixture must be rigorously oxygen free.

For the first time it has been possible with resting suspensions to demonstrate an actual synthesis of acetate from hydrogen and carbon dioxide. In the Warburg experiments conducted with actively motile washed cells, it was found that the carboxy-hydrogen reaction is unbelievably sensitive to the slightest traces of oxygen. The production of acetate from dextrose is lowered to about one-half its normal amount, whereas the carboxy-hydrogen reaction was completely stopped, when traces of oxygen were present.

BIOCHEMISTRY

STUDIES ON NUCLEIC ACIDS, ENZYMES, AND ENERGY-TRANSFER SYSTEMS

W. E. Cohn (Leader)
D. G. Doherty — M. Helen Jones
E. Volkin — J. X. Khym
B. L. Strehler — E. A. Lloyd
F. Vaslow — E. Jane Beale

Composition and structure of nucleic acids. (Cohn, Volkin, Khym, Jones) This is proceeding at present chiefly through the work on identification of the products of enzymatic hydrolysis reported here.

The products of enzymatic hydrolysis of nucleic acids. In view of the success of the enzymatic hydrolysis procedure of Klein, using desoxyribonuclease and intestinal phosphatase, in hydrolyzing DNA to identifiable nucleotide end products, it was decided to apply the same procedure to RNA (substituting ribonuclease for desoxyribonuclease). It was known from previous work in this laboratory that ribonuclease liberated cytidylic and uridylic acids as the only mononucleotide end products; it was hoped that dephosphorylation of the residual polynucleotides might facilitate their isolation and identification.

The results of this experiment, in which 5′-nucleotides were isolated from RNA digests for the first time, were very striking. A letter describing them (quoted in its entirety) has been accepted by Nature for early publication.

NUCLEOSIDE-5′-PHOSPHATES FROM RIBONUCLEIC ACID

Waldo E. Cohn and Elliot Volkin

"Though the manner in which the four nucleotides of ribonucleic acid (RNA) are combined in the intact molecule is not completely settled, the data obtained from analyses of the chemical degradation products are most compatible with phosphoryl group attachments through the C_2 and C_3 positions of the ribose group (Levene and Bass, "Nucleic Acids," Chemical Catolog Co., 1931; Gulland, J. Chem. Soc., 1938; Tipson, "Advances in Carbohydrate Chemistry," Academic Press, Inc., 1:193, 1945). The exclusion of C_5 phosphoryl linkages in RNA rests principally upon the failure to isolate 5′-nucleotides or the

acid-stable ribose-5-phosphate from acid hydrolysates of RNA. However, Gulland and Jackson (J. Chem. Soc., 1492, 1938), hydrolyzing yeast RNA with a venom containing a phosphodiesterase and a specific 5'-nucleotidase, found 35 per cent of the total phosphate liberated as inorganic phosphate, suggesting that a large number of the phosphoryl groups were attached to the ribose C_5 position. Recently, Schmidt (in press), utilizing periodate titrations of ribonuclease-hydrolyzed yeast RNA, concluded that some nucleotide groups had been linked to positions other than ribose C_2 or C_3 in the original RNA. The experiments described herein indicate that intact 5'-nucleotides can be isolated enzymically from calf liver RNA."

"Calf liver RNA, prepared by a technique avoiding the use of alkaline solutions (Volkin and Carter, J. Am. Chem. Soc., in press), was hydrolyzed successively by crystalline ribonuclease and by alkaline intestinal phosphatase in the presence of sodium arsenate to inhibit excessive dephosphorylation of the derived nucleotides, as originally described by Klein (Z. Physiol. Chem., 218:164, 1933), for the enzymatic production of desoxyribonucleotides. The reaction was allowed to proceed until about 75-80 per cent of the total titratable phosphoryl groups were released, after which the digestion was stopped by the addition of formic acid to a concentration of 0.1 *N*. The nucleotide products of the digest were then isolated by the anion-exchange procedure described by Cohn (J. Am. Chem. Soc., 72:1471, 1950; *ibid.*, 2811)."

"Figure 7 shows the ion-exchange elution curve of the nucleotides isolated from the digest. The identification of the 5'-nucleotides indicated on the figure was made chiefly by comparing their properties with those of the corresponding synthetic 5'-nucleotides (we are indebted to Dr. D. M. Brown and Prof. A. R. Todd for generous samples of synthetic guanosine-5'-phosphate, uridine-5'-phosphate, and cytidine-5'-phosphate) as follows: (a) the ion-exchange elution positions are identical with those of the analogous 5'-nucleotides (and desoxynucleotides) and are readily differentiated from the "a" isomeric forms (Cohn, J. Am. Chem. Soc., 72:1471, 1950; *ibid.*, 2811; Carter, J. Am. Chem. Soc., 72:1466, 1950); (b) the spectrophotometric absorption characteristics of the isolated pyrimidine-5'-phosphates show definite differences from those of the "a" and "b" forms, but are exactly those of the corresponding synthetic substances; (c) the isolated adenosine-5'-phosphate and muscle adenylic acid were both deaminated at exactly the same rate by a preparation of Schmidt's muscle adenylic acid deaminase (Kalchar, J. Biol. Chem., 167:461,

Figure 7

Analyses of (a) enzymatic digest and (b) alkaline digest of 20 mg of calf liver ribonucleic acid by ion exchange.

Exchanger: Dowex-1 (formate form), 200-400 mesh, 7 cm × 0.88 cm^2
Influent: Formic acid, sodium formate as shown; 0.6 ml/min

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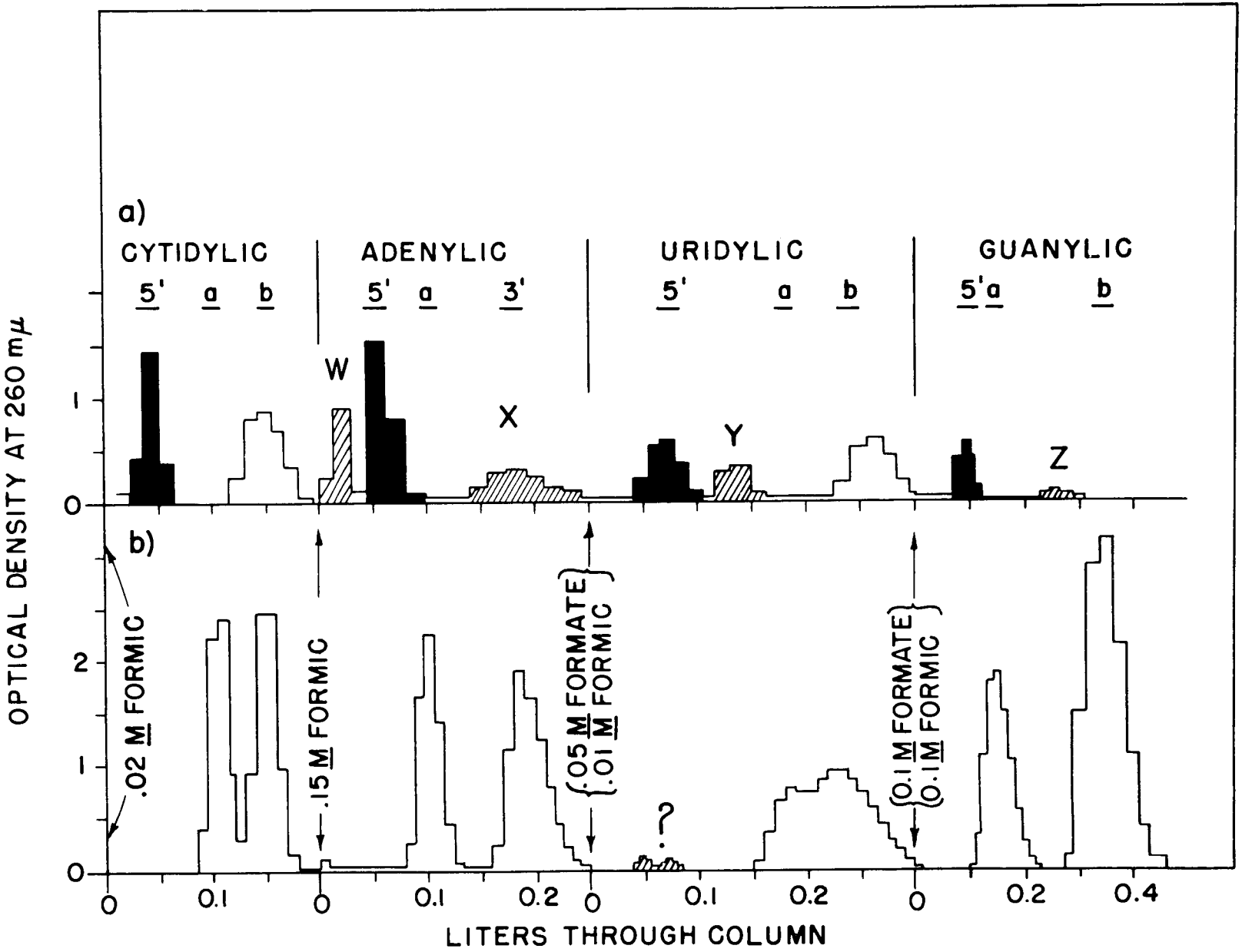

FIG. 7

1947), whereas adenylic acids "a" and "b" were unaffected by the enzyme; (d) a highly specific preparation of bull semen 5′-nucleotidase (kindly supplied by Dr. L. A. Heppel) readily and rapidly dephosphorylated all the isolated 5′-nucleotides, as well as the synthetic 5′ nucleotides, but was completely inactive toward all "a" and "b" mononucleotides; (e) the isolated 5′-adenylic acid contained adenine, pentose (orcinol reaction), and phosphate in a 1:1:1 proportion. Desoxypentose (diphenylamine reaction) was not present in any of the 5′ compounds nor was acid-labile phosphate (1 *N* hydrochloric acid, 1 hour, 100° C)."

"Enzymatic resynthesis of smaller fragments, rearrangements of mononucleotides to yield the 5′ compounds during the enzymatic hydrolysis and preexistence of the free 5′ compounds were excluded by the following experiments:

1. A similar enzymatic hydrolysis of RNA carried out in the presence of isotopic phosphate yielded 5′- and 3′-nucleotides containing none of the isotope (nor was arsenate incorporated).
2. Incubation of a mixture of the nucleosides or nucleotides with the phosphatase in a phosphate medium resulted in no measurable synthesis of any of the 5′-nucleotides nor ribose-5-phosphate.
3. Acid and alkaline hydrolysates yielded neither 5′ nucleotides nor ribose-5-phosphate.
4. Ribonuclease alone or ribonuclease followed by alkaline hydrolysis yielded no 5′-nucleotides; these appeared only after phosphatase treatment.

Thus, if isomerization during digestion does indeed take place, it must occur at the polynucleotide level."

"It is our intention to use ion-exchange chromatography, already responsible for the isolation of many new nucleotides (Cohn, J. Am. Chem. Soc., 72: 1471, 1950; *ibid.*, 2811) from nucleic acid hydrolysates, to determine the generality of this finding as well as in the search for hitherto unknown constituents, e.g., W, X, Y, Z, and ? in Fig. 7."

Following up this work, we have repeated the experiments of Gulland and Jackson (J. Chem. Soc., 1492, 1938) with snake venom which hydrolyzes only the 5′-nucleotides. This work is incomplete but it has shown us that calf liver RNA is completely hydrolyzed to low molecular-weight substances, with two-thirds of the phosphate released as inorganic phosphate (confirming, in essence, Gulland and Jackson), practically all the purines and two-thirds of the

pyrimidines as nucleosides, and the remainder of the pyrimidines as (apparently) cytidine diphosphate and uridine diphosphate. We are in the process of identifying these substances and following up the observation with parallel experiments on other nucleic acids. Our current opinion is that the combination of specific enzymes with the ion-exchange analytical technique may prove a major weapon in the elucidation of nucleic acid structure.

Work on the ion-exchange behavior of polynucleotide material, preliminary to devising practical separation schemes, indicates that the weak-base anion exchangers may be useful for this purpose. There remains the question of rate of reaction which, if too slow, could render impractical a separation scheme based upon equilibrium distribution coefficients alone. Along this line, the effects of mesh size, salt form, solvent, and temperature will be investigated for their influence upon rate.

The weak-base anion-exchange studies have indicated that these substances may prove to be of great use in the concentration steps of preparative procedures in that nucleotide material may be eluted from them with easily removed ammonia instead of, in the case of strong-base material, hydrochloric acid which, when concentrated, hydrolyzes the preparation.

Enzyme-substrate equilibria. (Vaslow, Doherty) In order to more closely define the nature of the active center of α-chymotrypsin a study of the binding of specific competitive inhibitors was initiated. The strongest competitive inhibitor closely allied to the substrate N-acetyl-3,5-dibromo-L-tyrosine would be 4-(4′-hydroxy-3,5-dibromophenyl)-3-acetamino-butanone-2 in which a methyl group is substituted for the carboxylic hydroxyl group. The most desirable synthesis was found to be the direct bromination of 4-(4′hydroxyphenyl)-3-acetamino-butanone-2. This compound had been previously prepared, but with widely different melting points and properties, by Levene (165° C), Dakin (135° C), and Neurath (116° C) (Levene and Steiger, J. Biol. Chem., 79:96, 1928; Dakin and West, *ibid.*, 78:91, 1928; Kaufmann and Neurath, *ibid.*, 181:623, 1949). It was prepared here by three procedures, the products obtained agreeing with Levene's preparation. This ketone was brominated by several methods, the most successful being the bromination in pyridine with pyridine perbromide hydrobromide. Enzymatic studies established this compound as a strong competitive inhibitor.

The procedure for bromination of the ketone with Br^{82} on a micro scale was developed based on the above macro procedure. Several experimental details were changed to yield a pure labeled pyridine perbromide hydrobromide and a pure labeled ketone for the enzymatic studies.

Some attempts have been made to develop a theoretical curve for the binding of N-acetyl-3,5-dibromo-L-tyrosine by α-chymotrypsin as a function of *p*H and ionization of several of the ionizable groups of the protein molecule. An assumption is made that the substrate is bound by a single ion or a specific combination of protein ions and not by others. The substrate binding constant may vary for each ion. The concentration of the enzyme-substrate complex may then be calculated from the mass action law. Simultaneous equations for the values of the binding and ionization constants were solved using the experimental binding and *p*H data. The fit for equations involving fewer than four parameters was poor, while one of the constants of the four-parameter equation was negative and therefore invalid. Addition of an electrostatic work term to a single constant equation did not particularly improve the fit. Since ionization of the protein is quite complex in the region of maximum enzymatic activity, further data seems necessary before the function of the hydrogen ion can be determined.

Studies on biological energy-transfer systems. (Strehler) 1. *Construction of a new quantum counter and preliminary experiments.* During the past quarter, a quantum counting apparatus (similar to that of J. A. Ghormley, Chemistry Div., this Laboratory) has been constructed for use in luminescence studies. This apparatus consists of a photomultiplier (1P22) operated at liquid nitrogen temperatures to reduce thermal electrons, an A-1 amplifier, and preamplifier which drives a scaler and logarithmic count-rate meter. Physically, the photomultiplier is mounted in a separate light-tight box in such a way that experimental setups may be arranged in an attached light-tight box without exposing the photomultiplier to room lights. Arrangement is also made for adding liquid nitrogen to the photomultiplier without exposing it to light. The apparatus has been put to the uses mentioned in the following paragraphs.

Luminol (3-aminophthalhydrazide) and dimethyl diacridylium nitrate were synthesized and the former was used to determine oxygen tension in a flowing gas mixture preparatory to using this method for determining oxygen evolution

in photosynthesis. Preliminary studies indicated that this method is quite sensitive to oxygen, tank nitrogen giving appreciable counts. Hemin as an oxygen activator was added to an alkaline solution of 3-aminophthalhydrazide. Figure 8 illustrates the effect of tank nitrogen and air on luminescence.

In cooperation with C. W. Sheppard (Radiobiochemistry), experiments were done on determination of adenosinetriphosphate (ATP) content of human erythrocytes. Preliminary studies on erythrocytes from which ATP was extracted at various times after irradiation indicate no pronounced difference in ATP concentrations in irradiated and nonirradiated cells up until 4 hours after irradiation.

In another preliminary study it has been shown that illuminated chloroplasts produce ATP or some other factor which results in an increased light output by the firefly enzyme.

2. *Studies on the luminescence of green plants.* Work has been begun on the luminescence of isolated chloroplasts and crude leaf extracts, first observed some time ago in this laboratory. Some fifteen representative species of plants, furnished through the courtesy of Dr. L. F. Bailey of the Biology Department, University of Tennessee, have been tested for luminescence after illumination. All plants tried have exhibited the phenomenon. For leaf homogenate studies, spinach and mustard greens have been used. Figure 9 illustrates the decay curves of mustard green whole leaves and homogenate.

A low steady-state luminescence of about 80 counts per minute was observed in crude leaf extract for as long as 35 minutes after illumination. Washing the chloroplasts caused a decay to near background after some 10 minutes while whole leaves decayed to zero after 5 minutes.

The effect of *p*H on the luminescence of mustard green extract has been determined and appears in Fig. 10. The two optima may indicate that two different systems are involved in either absorption or emission of the light.

In an attempt to determine whether luminescence and some process known to be related to photosynthesis saturate at the same light intensity, the rate of the Hill reaction in isolated chloroplasts was determined using the direct potentiometric method recently developed by Spikes, Lumry, Eyring, and Wayrynen (Arch. Biochem., 28:48, 1950) at the same time as light emission measurements were made. The apparatus used in these experiments consisted of a constant

EFFECT OF TANK N_2 AND AIR ON LUMINESCENCE OF LUMINOL

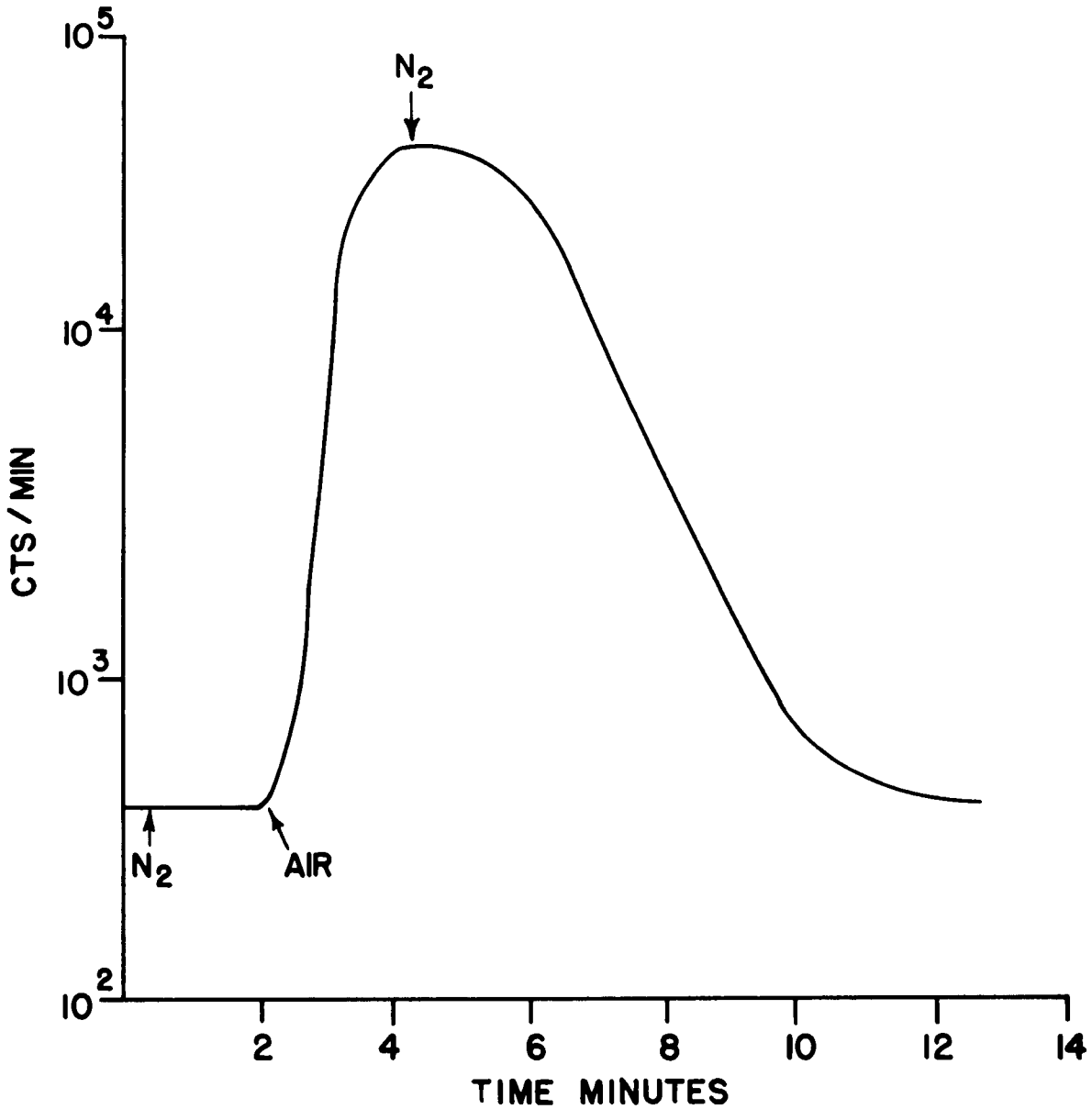

FIG.8

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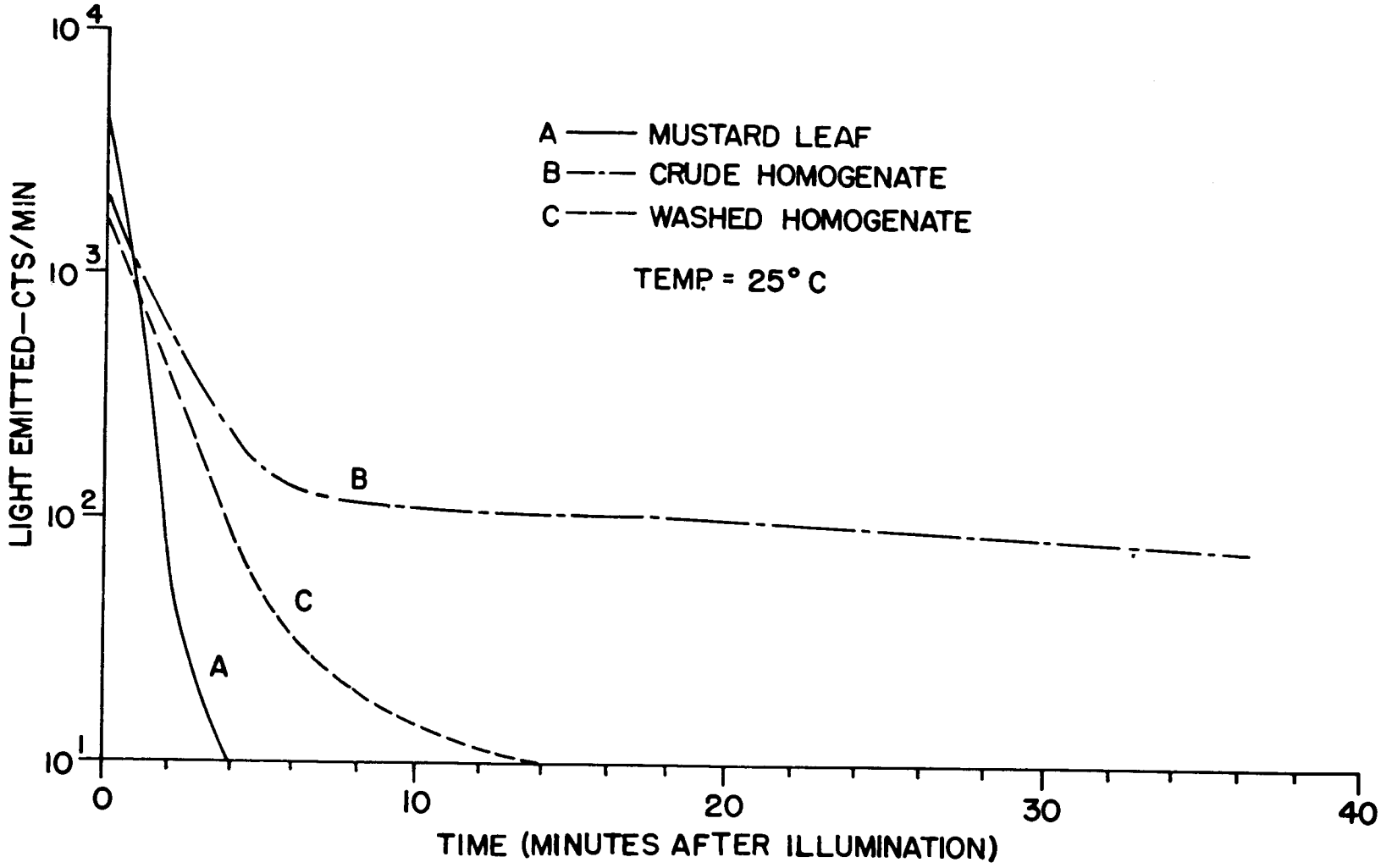

FIG.9

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MUSTARD CHLOROPLAST pH ACTIVITY CURVE

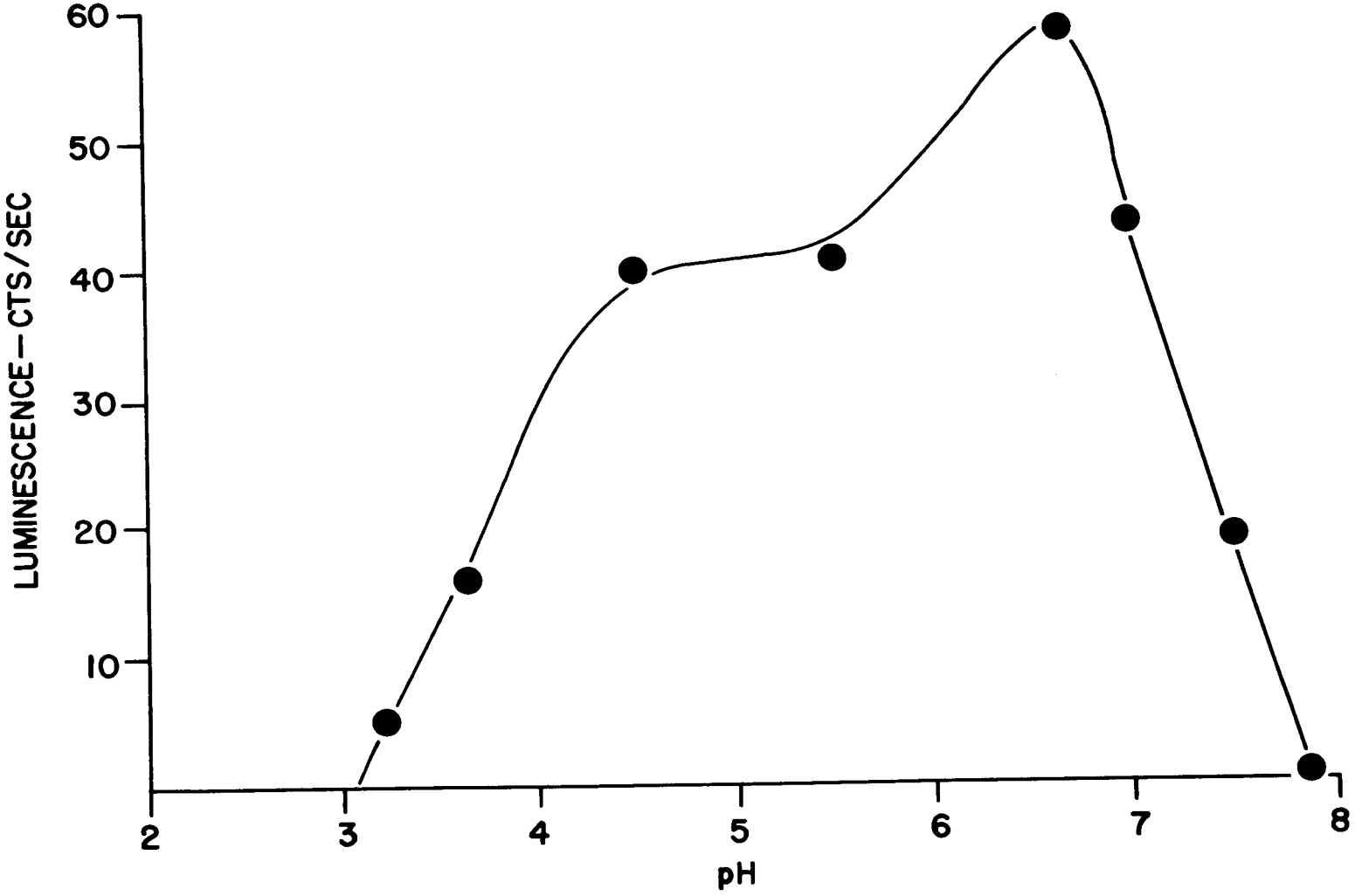

FIG. 10

temperature circulating system which could be illuminated at different intenties. Arrangement was made for the measurement of potential changes and luminescence values as functions of the exciting light and for recording these values directly on a two-point brown recorder. Figure 11 was obtained from the data on such a chart. The slope of the potentiometer change (rate of reduction) on illumination was plotted as a function of the intensity of illumination, as was luminescence. From Fig. 11 it can be seen that luminescence and the Hill reaction saturate at about the same light intensities and that the rates parallel each other over the entire range of illumination investigated. It is felt that these results are significant in establishing the direct relationship between rate of photosynthesis and rate of luminescence and will make it possible to measure photosynthetic rates under a variety of conditions in a much more simple manner than heretofore.

The work on *Chlorella* luminescence, in collaboration with W. A. Arnold (Biophysics) has been completed. The action spectrum for *Chlorella* luminescence has been redetermined with improved optics; within the experimental error, it is identical with the action spectrum for *Chlorella* photosynthesis as determined by Emerson *et al.*, (Emerson and Lewis, Am. J. Botany, 30:176, 1943).

3. *Studies on one-carbon metabolism of Neurospora* (with J. R. Totter). Mutant 8043-M, a strain of *Neurospora,* earlier described by the author, which requires either P-aminobenzoic acid (PABA) or methionine for growth was studied under various conditions with C^{14} methyl-labeled betaine and α-C^{14}-labeled glycine. It was found, contrary to expectations, that betaine is not utilized by *Neurospora* at physiological *p*H. Glycine, on the other hand, is rapidly utilized, but the overwhelming mass of counts either in the presence or absence of PABA in either resting-cell preparations or growing cultures is found in serine. Practically no counts appeared in methionine. This implies that another methyl donor is functioning in *Neurospora* for methionine synthesis.

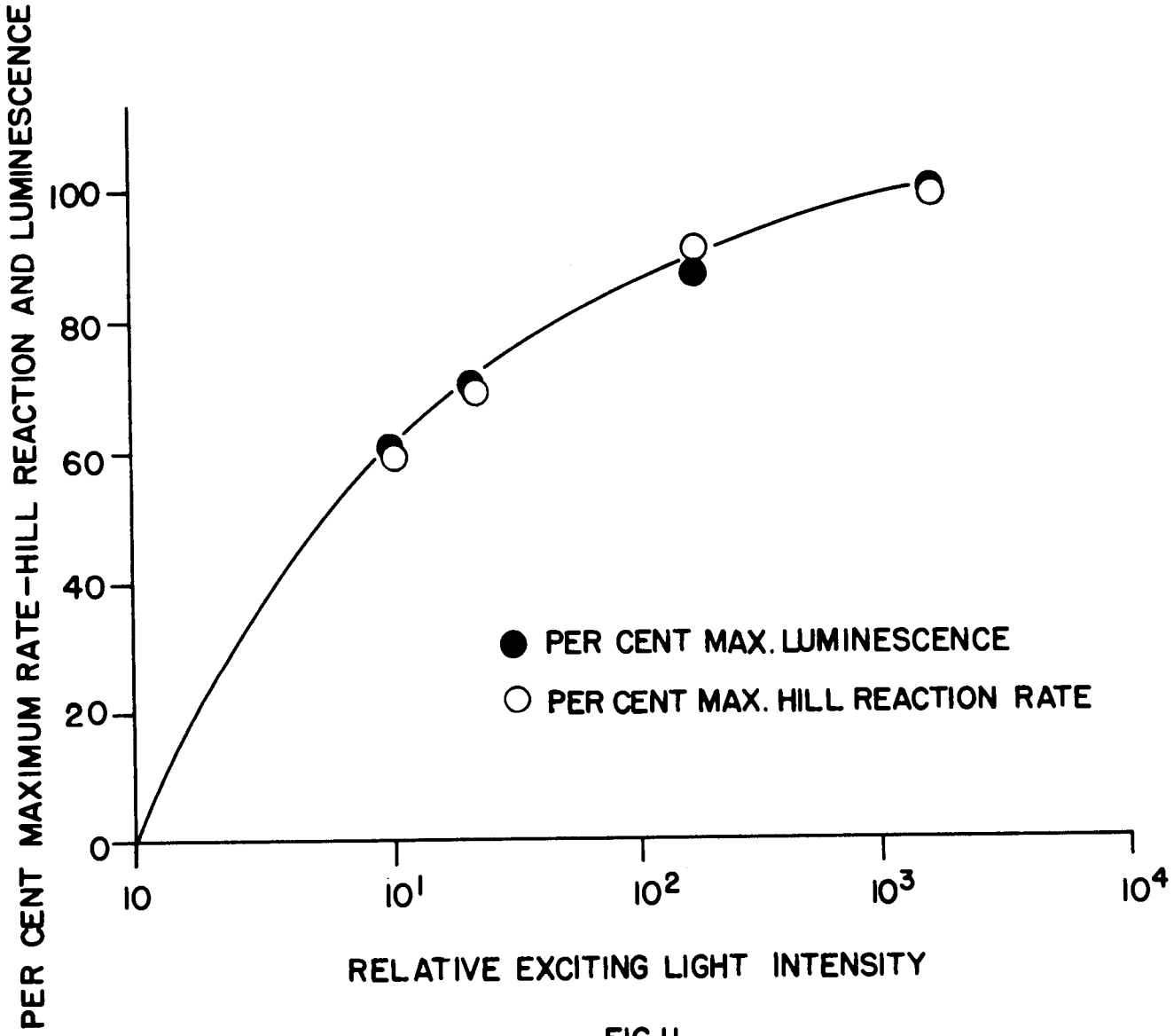
PER CENT MAXIMUM RATE-HILL REACTION AND LUMINESCENCE
100
80
60
40
20
0
10
10¹
10²
10³
10⁴
RELATIVE EXCITING LIGHT INTENSITY
PER CENT MAX. LUMINESCENCE
PER CENT MAX. HILL REACTION RATE

FIG. 11

RADIOBIOCHEMISTRY

C. W. Sheppard (Leader)
W. T. Burnett, Jr. E. B. Darden, Jr.
J. S. Kirby-Smith Marian Stewart
Arthur Burke M. L. Morse
(See also Cytogenetics - Hollaender)

Erythrocytes. (Sheppard and Stewart) The effect of varying the oxygen tension of erythrocytes on their sensitivity to radiation-produced potassium loss has been investigated and the results will be presented at the Federation meeting in April. An acceptable method has been developed for equilibrating blood under atmospheres containing 5 per cent carbon dioxide plus oxygen or helium. By spectrophotometric measurements on the hemoglobin of the cells it was determined when complete equilibrium was reached. The method avoids the usual danger of injuring the cells by foaming, and does not require their removal from the plasma. Using this procedure the potassium leakage following exposure of the blood to 35,000 to 40,000 r has been compared in samples saturated with oxygen and with helium. The leakage in helium was found to be greater, if anything, than that in the oxygenated blood, although the differences were not considered significant. This places the erythrocyte disturbances in a different category from those in other materials which show the now familiar oxygen effect.

In view of the observations by Greig and Holland of the relation between acetylcholine turnover and potassium accumulation, an attempt was made to develop a method for concentrating the acetylcholine in blood using an ion-exchange method. It was hoped that in this way chemical determinations could be made which were specific not only for esters but also for cations, thus increasing the specificity of the method of Hestrin for determining acetylcholine. The resin method would also make it possible to estimate the exceedingly minute amounts of this material in blood by concentrating it from a larger volume. Unfortunately the investigation was discontinued after three weeks since no acceptable procedure was found to prevent the spontaneous hydrolysis of the ester in the presence of the resin surface. Dr. Greig, of the Department of Pharmacology at Vanderbilt School of Medicine, has agreed to assist in the determination of acetylcholine by the classical effect on the frog rectus muscle.

Some time was spent during the last quarter in developing methods for studying the relation between sugar utilization and lactate production in irradiated blood. In addition, a joint study has been in progress during part of the period with Strehler (Energy-Transfer group) on the ATP content of irradiated and control cells. In this work he is successfully applying the luminescence technique for determining this compound, which is so important in its role as a potential cellular energy source.

Radiological physics. (Darden) Tests have been completed on the condenser type beryllium ion chamber qualifying it as a satisfactory instrument for the measurement of gamma radiation in the presence of thermal neutrons. In the thermal column studies begun last summer various gases such as oxygen, nitrogen, and helium as well as air were used in the chamber to see if the n-p reaction in nitrogen was an important factor. The saturation characteristics of these gases (nitrogen excepted) was studied using cobalt gamma rays as the reference radiation. By measuring with a vibrating reed electrometer the ion current generated (with potentials of from 1 to 500 volts on the chamber) with air and oxygen, the current remained essentially independent of the voltage over the electrometer operating range, as expected; with helium, the anomalous behavior experienced in the thermal column and cobalt radiation measurements was reflected by a complete breakdown of saturation ability above about 80 volts.

In order to reduce the rate of insulator leakage that occasionally caused inconvenience in making measurements with the chamber, fluorethene insulators were tried in place of teflon. Incomplete tests in the thermal column indicated that, at least for short exposure intervals, the substitution was satisfactory and the leakage rate was reduced appreciably.

RESULTS AND CONCLUSIONS

The gamma radiation can be satisfactorily measured by Be chamber method. Contribution due to the n-p reaction in the air of the chamber amounts to about 10 per cent of the total intensity measured. The total intensity based on calibrations with cobalt gamma roentgens ranges from about 8 to 11 r/minute in the graphite box with it in the treatment position inside the bismuth safe, depending on the point of measurement in the box. This is for a normal winter operating power level. The intensity roughly parallels corresponding flux

measurements made in the box. The gamma-ray intensity falls off rapidly inside the box at points a few cm above the bottom position and is down by the factor of 2 within half a meter. The contribution to the intensity due to capture radiation from the graphite of the column and due to pure bismuth is too small to be significant. Lead absorption measurements made in the boron plastic box which agree in general with those made previously by Stapleton with a Victoreen chamber indicate that the gamma radiation has an energy of from about 2½ to 5 mev. The presence of the boron causes about a 40-per cent reduction in the measured intensities compared to the results inside the graphite box when the soft gamma-ray contribution due to the boron itself is subtracted. It is concluded that a contaminant of high-capture cross section present in the vicinity of this bismuth safe, or in the bismuth itself, is responsible for the bulk of gamma radiation.

Work has been started on measuring depth dose in tissue equivalent materials due to beta rays from P^{32} bakelite plaques using our standard extrapolation chamber. It is planned in this way to refine some of the measurements of depth dose made here earlier by Raper, Zirkle, and Barnes.

Modification specifications have been submitted which should enable the chamber to be used for measuring the surface intensity of somewhat smaller metallic plaques containing ruthenium developed by the Isotopes Division. These plaques provide a much longer lived source of radiation than the P^{32}.

Infrared studies. (Kirby-Smith) Preliminary work toward a study of the effects of combined infrared and X radiations on *Tradescantia* microspores has been completed. This has included the assembly of infrared irradiation equipment as well as a program of familiarization with the methods of pollen tube culture.

Exploratory experiments using an infrared image tube in conjunction with an ordinary microscope have shown that biological material may be easily studied in the region of 7000 to 12,000 A. Further work in the possible application of this technique is in progress.

PLANT PHYSIOLOGY

G. R. Noggle (Leader)
J. H. Taylor* G. M. Cheniae
L. P. Zill M. Eleanor Schumacher
J. S. Bangson**

Plant biosynthesis. (Noggle, Schumacher). Considerable work has been done during the past quarter on working out a satisfactory degradation scheme for the position of C^{14} in biosynthetically prepared sugars. The method of Wood, Lifson, and Lorber (J. Biol. Chem., 195:457-489, 1945) has been used extensively for glucose degradation and was first tried. The glucose is fermented to lactic acid with a suspension of *Lactobacillus casei*. The lactic acid is then isolated and degraded by chemical methods.

The problem of getting a uniform suspension of *L. casei* was considered first. After some preliminary trials the following media were found satisfactory.

Glucose	1 per cent
Yeast extract	1 per cent
Sodium phosphate	5.69 g/liter
Potassium phosphate	3.63 g/liter

The sodium and potassium phosphate buffers the solution to *p*H 6.9. The *L. casei* was kept on agar slants (0.5 per cent glucose, 1.5 per cent yeast extract, 1.5 per cent agar) and then transferred to 10 cc of broth. After 48 hours the 10-cc culture was poured into a 60-cc flask of broth. The contents of this flask were stirred continuously for 48 hours and then transferred to 600 cc of broth. After 48 hours of continuous stirring the cells were centrifuged out and washed three times with distilled water. These cells were then used to ferment glucose to lactic acid.

Following fermentation the cells were centrifuged and supernatant decanted off and made alkaline to phenol red with 0.1 *N* sodium hydroxide. The supernatant was then ether extracted for 24 hours. The solution was then adjusted to a *p*H of 1.0 with 50 per cent sulphuric acid and extracted with ether for 18 hours. The ether for these extractions was alkali and acid washed. Following the acid ether extraction, which extracts lactic acid from

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**Research Participant

the fermentation mixture, the ether was evaporated off and the solution steam distilled. The lactic acid remains behind and is then ready for chemical degradation.

In order to check on the purity of the lactic acid the samples were partitioned on a Celite column with ethyl ether as the mobile phase and 0.5 *N* sulphuric acid as the inside phase. A column 1 cm in diameter and 40 cm long was used and at a flow rate of 0.6 cc per minute the lactic acid appeared in the eluate after about 60 cc of ethyl ether had passed through. Acetic acid is found after about 20 cc of ethyl ether has passed through the column. The partition mixture of ethyl ether and 0.5 *N* sulphuric acid will not separate α-ketoglutaric acid and lactic acid. In order to test for the presence of α-ketoglutaric acid in the lactic acid fraction a test was run with 2,4-dinitrophenylhydrazine. Several times a precipitate was noted after the addition of the 2,4-dinitrophenylhydrazine which indicated that α-ketoglutarate might be present. These positive tests were observed during the preliminary work on the fermentation method but after the procedure had been stardardized no positive tests were found. The only impurity found in the lactic acid sample is acetic acid. Acetate should be removed by steam distillation but evidently the separation is not complete. It was also found that if unwashed ether was used during the partition chromatogram a high acetate value was obtained. When the ether was alkali and acid was washed, low acetate values were found.

As a matter of routine procedure all sugar fermentations are partitioned in a Celite column so as to obtain pure lactic acid for subsequent degradations. Figure 12 gives the results from the partition chromatogram of the lactic acid obtained from the fermentation of 100 mg (0.56 m*M*) of glucose. The lactic acid was obtained by an alkaline ether and acid ether extraction followed by steam distillation. The residue from the steam distillation was titrated to the phenol red end point with 0.1 *N* sodium hydroxide. This was equivalent to 12.34 cc. The sample was then passed through a Celite column with ethyl ether and 0.5 *N* sulphuric acid. The lactic acid accounted for from the column was 0.852 m*M*; theoretical yield was 1.1 m*M*. The results from the partitioning of a number of known lactic samples showed that approximately 85 per cent of the lactic acid could be recovered. The extra 0.1 *N* sodium hydroxide needed to titrate the lactic acid sample was used to titrate sulphuric acid that was extracted by ether from the fermentation mixture.

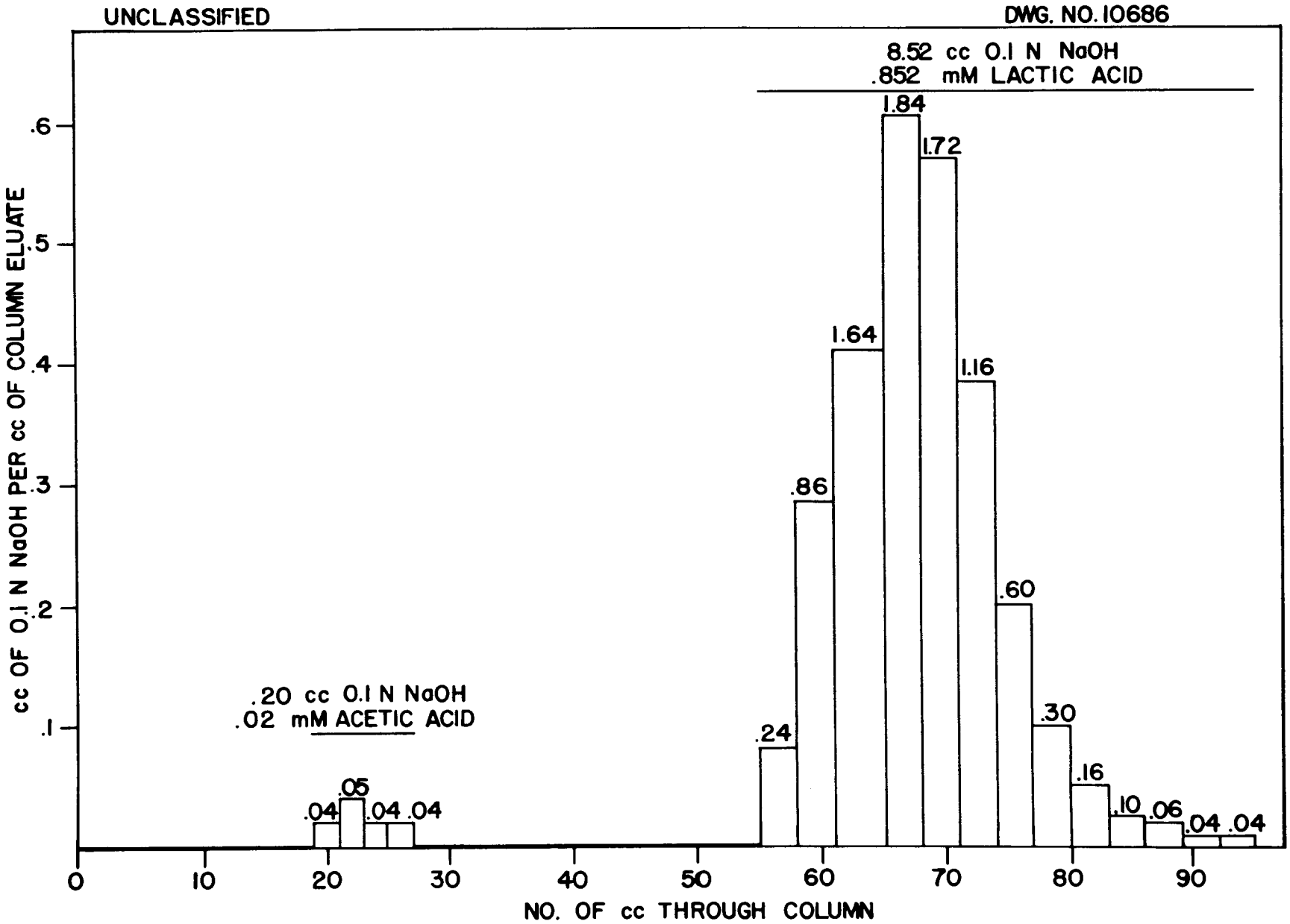
UNCLASSIFIED
DWG. NO. 10686
8.52 cc 0.1 N NaOH
.852 mM LACTIC ACID
.20 cc 0.1 N NaOH
.02 mM ACETIC ACID
.04
.05
.04
.04
.24
.86
1.64
1.84
1.72
1.16
.60
.30
.16
.10
.06
.04
.04
cc OF 0.1 N NaOH PER cc OF COLUMN ELUATE
.1
.2
.3
.4
.5
.6
0
10
20
30
40
50
60
70
80
90
NO. OF cc THROUGH COLUMN

FIG. 12

Figure 13 shows the results obtained from the partition chromatogram of the lactic acid resulting from the fermentation of 100 mg of fructose. The residue following steam distillation titrated 11.64 ml 0.1 *N* sodium hydroxide. The lactic acid from the column amounted to 0.681 m*M*.

The lactic acid was decarboxylated by cold potassium permanganate. The carbon dioxide was converted to barium carbonate and counted for activity in the 3,4-positions of glucose. The acetic acid was recovered by steam distillation and degraded as described by Mosbach, Phares, and Long (ORNL-889) for activities in the 2,5- and 1,6-positions of glucose.

Studies on the physiology of Lilium longiflorum anthers. (Taylor, Noggle) Work has continued on the problem that was described in the ORNL-807. The recent work has been concerned with the nutrition of lily anthers in culture. Excised lily anthers have been grown in a variety of media containing mineral salts, vitamins, indole acetic acid, sucrose, amino acids, and coconut milk. These have been used in concentrations and combinations that have proved successful in the culture of various callus type or tumor tissues of plants. Most of these combinations have shown some stimulation on the growth of the vegetative portions of the anthers but usually at the expense of the sporogenous tissue which continues normally for 2 or 3 days in culture solutions. The sporogenous tissue continues its normal development for longer periods in media that contain but few added nutrients, indicating a rather low metabolic rate for meiosis particularly from zygotene through the two divisions. Cultures from earlier stages have not resulted in the completion of meiosis. Spindle formation remains normal when all cleavage and wall formation fails in those anthers that develop without additional nutrients. This suggests that the energy requirements for spindle formation and chromosome movement must be relatively low.

There is some indication from the culture experiments that glutamic acid is utilized, but that very little sucrose is utilized during meiosis. The uptake of C^{14}-labeled sucrose, as measured by the activity in the alcoholic extract of anthers after 3 days in culture, was about equal to the activity of the C^{14}-labeled sucrose culture medium. This may be due to a differential uptake of C^{12}- and C^{14}-labeled sucrose. In older anthers, in which pollen grain walls were forming, the concentration of C^{14}-labeled sucrose was six times that of the culture medium.

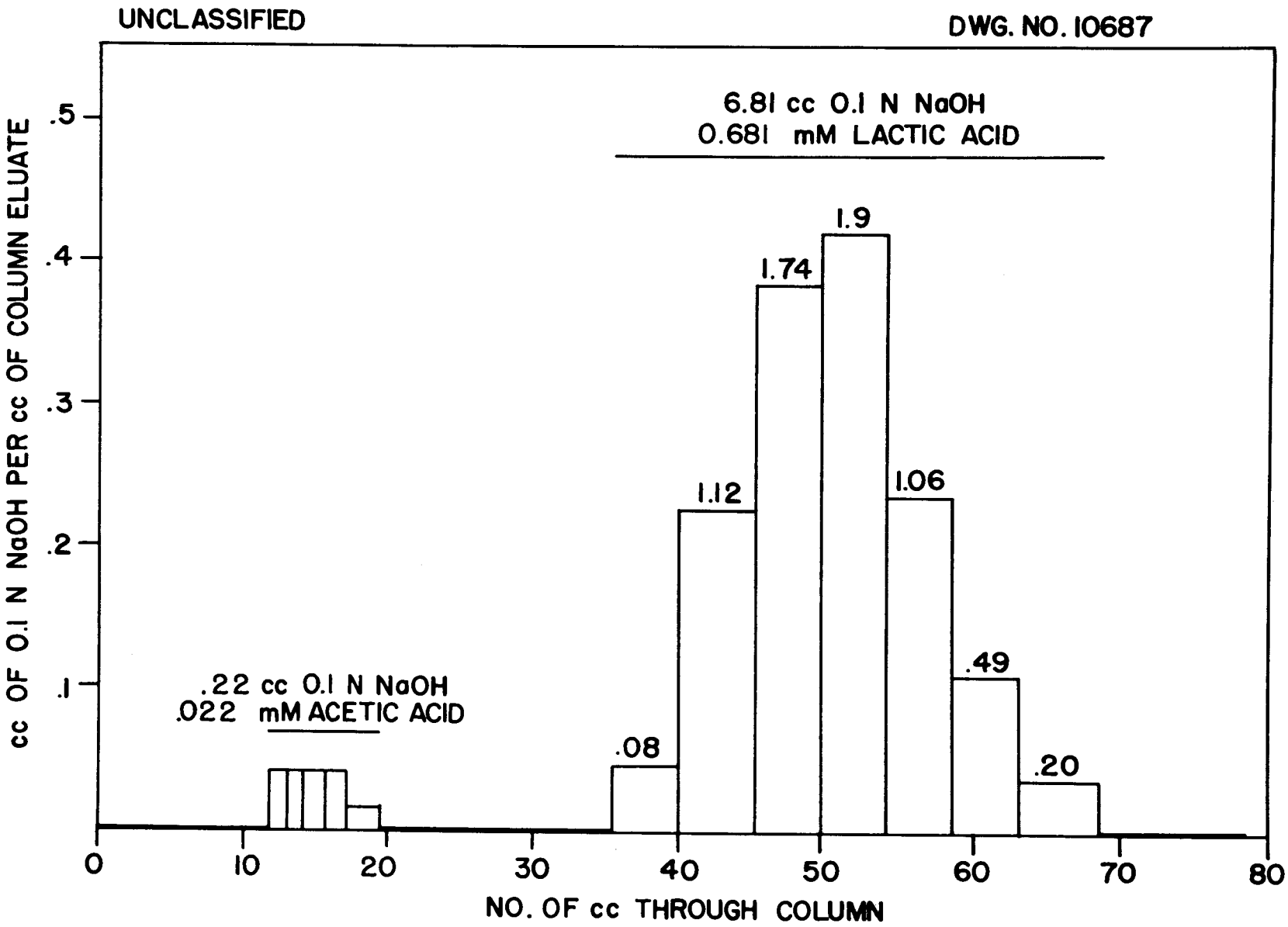
UNCLASSIFIED
DWG. NO. 10687
6.81 cc 0.1 N NaOH
0.681 mM LACTIC ACID
.22 cc 0.1 N NaOH
.022 mM ACETIC ACID
.08
1.12
1.74
1.9
1.06
.49
.20
.5
.4
.3
.2
.1
0
10
20
30
40
50
60
70
80
cc OF 0.1 N NaOH PER cc OF COLUMN ELUATE
NO. OF cc THROUGH COLUMN

FIG.13

Infiltration with radioactive nutrients and estimation of the concentration of the chemically bound quantities in various portions of the anther by radioautographs may prove a valuable technique for the study of the metabolism or turnover rates. For example, a series of stages in meiosis from pachytene through metaphase I may be found in a progressive series along the length of one anther. The ratio of activity in the regions that include different stages of division after short periods of infiltration and metabolism should give a measure of turnover rate for the particular substance during or just preceding the stages fixed. Since these cells of the sporogenous tissue are so highly synchronized the measurements could be calculated in terms of single cells. Preliminary attempts with C^{14}-labeled sucrose indicate that such measurements can be successful for any substance that can be infiltrated rapidly and utilized after short periods. Phosphorus should be tried next since its turnover rate is so closely related to nucleic acid synthesis and energy-yielding metabolism.

Yeast biosynthesis. (Zill) The investigation of yeast as a source of labeled trehalose was continued. As previously reported (ORNL-889), the yield of trehalose obtained from *Torulopsis utilis* var. major was less than 1 per cent of the dry weight of the yeast. The method of isolation was checked by isolating the trehalose from 205 g of Fleischmann's active dried yeast (Standard Brands, Inc.) which has been reported to contain *ca* 16 per cent of the disaccharide. The yield was 31 g of crystalline trehalose or 15 per cent of the dry weight, which is in good agreement with the reported yield. It has been shown that both the method of culture and the drying procedure are critical factors in the production of yeast with a high trehalose content. However, such procedures have not been published since they constitute operational factors of a highly commercial nature.

Stock cultures of the yeast used for the preparation of active dried yeast were obtained through the courtesy of Mr. R. F. Light (Fleischmann Laboratories, Inc.). This yeast was used (as was also *T. utilis* var. major) in several attempts to increase the yield of trehalose. Included in these attempts was the growth of the yeast on a nitrogen-deficient medium without aeration followed by lyophilization, or drying *in vacuo*, at 30° C. Yields of less than 1 per cent trehalose were obtained in all experiments.

The low yields of trehalose obtained do not detract from the value of yeast as a source of labeled glucose since yeast glycogen, which is present in

much higher amounts than trehalose, represents an even better source of the sugar than does trehalose. The ordinary procedure for the isolation of glycogen involves the use of hot concentrated alkali as a first step. For our purpose it was desirable to dispense with this drastic procedure since we wish to isolate the ribonucleotides from the residues remaining after extraction of the glycogen. It was found that repetitive extraction with 5 per cent trichloroacetic acid resulted in almost complete extraction of the glycogen. The glycogen obtained from yeast in this manner is always accompanied by a large amount of yeast mannan which exhibits properties very similar to the glycogen. After preliminary purification of the glycogen-mannan mixture, the mannan may be separated from the glycogen by means of an insoluble copper-mannan complex produced by means of Fehling's solution. The mannan-glycogen mixture may also be first hydrolyzed and then separated by means of a chromatographic column technique (G. R. Noggle).

A mixture of *ca.* 4 g of reducing sugars from 44 g of dried yeast has been obtained by this method. The separation of the sugars by the column technique is in progress at the present time. A second determination which is in progress indicates a larger yield due to a better extraction obtained after greater disintegration of the yeast cells (by prolonged ball-milling). By means of the above described procedures, not only labeled glucose but also labeled mannose may be obtained.

Thirty-three grams of yeast containing approximately 1650 μc of C^{14} have been prepared as a source of the labeled compounds. This yeast is to be diluted with nonlabeled yeast and processed for its content of glucose and mannose by the above described procedures and for its content of ribonucleotides by the procedure previously described (ORNL-889).

The growth of peanuts following irradiation with X and gamma rays. (Noggle, Bangson; Darden, of Radiobiochemistry) Additional data were obtained from the peanut experiment described in ORNL-807. Thirty days after planting information was gathered as to height of main stem, number of expanded leaves on main stem, and number of expanded leaves on the cotyledonary stems. The data were tabulated and turned over to Moshman of the Mathematics Panel for analysis. In Table 10 is given the plan by which the experiment was carried out; in Table 11, the data when examined by the analysis of various techniques.

TABLE 10

Dose by Source of Radiation Effect on Peanuts

				X	γ
1.	Let	A =	2,500	1	2
		B =	5,000	3	4
		C =	10,000	5	6
		D =	20,000	7	8
		E =	40,000	9	10
		F =	Control	11	12

2. Let odd numbers = X rays
even numbers = γ rays

3. Let plots = 2 rows 25 seeds/row (50 seeds/plot) across the greenhouse bench. Plant seeds 2.5 in. apart with rows 4 in. apart between rows, 1-1.5 in. deep.

4. Leave 12 in. at each end of bench and 5 in. between each end of the row and the edge of bench.

5. Plant 24 plots (48 rows) in each bench (2 repl./bench)

Experimental Plan - Replications

	I		II	III	IV	V	VI	VII	VIII
PLOT NO.	DOSE	ENTRY	P D E	P D E	P D E	P D E	P D E	P D E	P D E
1	D	7	13 B 3	25 A 2	37 D 7	49 F 12	61 E 9	73 B 3	85 F 12
2		8	14 4	26 1	38 8	50 11	62 10	74 4	86 11
3	C	6	15 C 5	27 F 11	39 B 3	51 E 9	63 B 4	75 E 10	87 D 8
4		5	16 6	28 12	40 4	52 10	64 3	76 9	88 7
5	E	9	17 F 11	29 C 6	41 E 10	53 A 2	65 D 8	77 A 1	89 C 5
6		10	18 12	30 5	42 9	54 1	66 7	78 2	90 6
7	F	11	19 D 8	31 E 9	43 F 12	55 D 8	67 F 12	79 F 11	91 A 1
8		12	20 7	32 10	44 11	56 7	68 11	80 12	92 2
9	A	1	21 E 9	33 D 8	45 A 1	57 B 4	69 A 2	81 C 6	93 E 10
10		2	22 10	34 7	46 2	58 3	70 1	82 5	94 9
11	B	4	23 A 2	35 B 4	47 C 6	59 C 6	71 C 6	83 D 7	95 B 3
12		3	24 1	36 3	48 5	60 5	72 5	84 8	96 4

TABLE 11

SOURCE OF VARIATION	DEGREES OF FREEDOM	LEAVES (main)			LEAVES (cotyledonary)			HEIGHT (main stem)		
		SUM OF SQUARES	MEAN SQUARE	F RATIO	SUM OF SQUARES	MEAN SQUARE	F RATIO	SUM OF SQUARES	MEAN SQUARE	F RATIO
Treatment (T)	1	1.291	1.291	16.99[a]	0.492	0.492	2.15	4.572	4.572	4.99[b]
Dose (D)	5	60.604	12.121	159.49[a]	377.242	75.448	329.47[a]	1719.051	343.810	375.34[a]
Bench (B)	3	5.349	1.783	23.46[a]	15.414	5.138	22.44[a]	62.651	20.884	22.80[a]
T x D	5	9.223	1.845	24.28[a]	1.012	0.202	0.88	8.277	1.655	1.81
T x B	3	0.339	0.113	1.48	0.427	0.143	0.62	0.858	0.286	0.31
D x B	15	2.303	0.54	2.02[b]	3.754	0.210	1.09	17.903	1.194	1.30
T x D x B	15	0.890	0.059	0.78	1.650	0.110	0.48	6.304	0.420	0.46
Error	48	3.652	0.076		10.986	0.229		43.960	0.916	
Total	95	83.651			410.977			1863.576		

a Significant on 0.001 level
b Significant on 0.05 level

There was a highly significant variation between X and gamma radiation with respect to the number of leaves on the main stem. The numbers of leaves on either the main stem or cotyledonary stem were not greatly affected by the various dose levels until 40,000 r was used. The height of the main stem showed decreases in growth at dose levels above 5000 r.