UCRL 8421

UNIVERSITY OF CALIFORNIA

Radiation Laboratory

BIO-ORGANIC CHEMISTRY QUARTERLY REPORT

March, April, May, 1958

BERKELEY, CALIFORNIA

DISCLAIMER

UCRL-8421
Chemistry–General

UNIVERSITY OF CALIFORNIA

Radiation Laboratory
Berkeley, California

Contract No. W-7405-eng-48

BIO-ORGANIC CHEMISTRY QUARTERLY REPORT

March, April, May 1958

June 27, 1958

Printed for the U. S. Atomic Energy Commission

Printed in USA. Price $1.50. Available from the
Office of Technical Services
U. S. Department of Commerce
Washington 25, D. C.

BIO-ORGANIC CHEMISTRY QUARTERLY REPORT

March, April, May 1958

Contents

*Preceding Quarterly Reports: UCRL-8204, UCRL-8141

BIO-ORGANIC CHEMISTRY QUARTERLY REPORT

March, April, May 1958

M. Calvin, Director
Edited by Edward L. Bennett

Radiation Laboratory and Department of Chemistry
University of California, Berkeley, California

June 27, 1958

DELAYED LIGHT EMISSION IN GREEN PLANT MATERIAL: TEMPERATURE DEPENDENCE AND QUANTUM YIELD

A paper of this title by Gordon Tollin, Eiji Fujimori, and M. Calvin is being prepared for publication in the Proceedings of the National Academy of Sciences, U. S., and being issued in preprint form as UCRL-8293, July 1958.

THE METABOLISM OF WATER BY CHLORELLA

V. Moses

A study is currently being made of the incorporation of tritiated water by Chlorella cells suspended in various media, and under conditions of light and of dark. This work is part of a program to study the path of hydrogen in photosynthesis.

An early program to use deuterated cells to follow the path of hydrogen in photosynthesis was abandoned when it was found that such cells showed distinct pathological characteristics[1] and that the quantities required for the detection apparatus (nuclear magnetic resonance) would involve the use of very large amounts of cell material, and would necessitate the isolation of intermediates on a large scale. In addition, deuterium is a stable species, and the techniques available for following the path of carbon by making use of the radioactivity of C^{14} cannot be applied to studies utilizing deuterium.

There remained the possibility of using tritium to follow the path of hydrogen from radioactive water in cells actively carrying out photosynthesis. Many difficulties had to be overcome, not the least of which were the very weak energy of the beta particles emitted by tritium, and the large dilution of the radioactive tracer by the relatively enormous amounts of water that are inevitably present in any biological system. The energy of the beta particles emitted by tritium averages about 18 kev, as compared with about 150 kev for those emitted by C^{14}. This means that the penetrating power of the particles is very much less than of those from carbon, and their ability to pass through paper and affect photographic film is correspondingly diminished.

[1] Moses, Holm-Hansen, and Calvin, Biochim. et Biophys. Acta 28, 62 (1958).

Detection of the radioactive materials on paper chromatograms becomes considerably more difficult for this reason, and to compensate in part for the weaker radiation, larger amounts of radioisotope have to be used. In systems in which the cells are supplied with radioactive carbon dioxide, the system can be so arranged that the supply of unlabeled carbon dioxide to the cells is reduced to a minimum, keeping the specific activity of the added tracer high. This is not possible with water; the only possibility is to use cell suspensions much more concentrated than those used for the carbon studies, in order to achieve a more favorable substrate-to-cell ratio. This in turn presented new difficulties; as the cell concentration in the suspension was increased, the optical density of the suspension rose very considerably and the amount of light passing through the suspension was correspondingly reduced.

The problem was finally solved in two ways. The conventional "lollipops" were abandoned in favor of small cylindrical vessels with flat bottoms, of such a size that 1 ml of liquid in them formed a layer on the bottom 1 mm deep. The vessels were shaken for the desired incubation period over a bank of fluorescent lights and the cell concentration was increased from the usual value of a 1% suspension (1 ml of wet-packed cells/100 ml of suspension) to a concentration of 12 to 25%. The second modification from the carbon work was to increase the dose of labeled tracer added from about 20 μC/ml in the carbon work to a specific activity in the tritium studies of 1 C of tritiated water per ml. In this way, some effort was made to overcome the disadvantages of the very weak radiation. Owing to the health hazards of working with such high specific activities of radiation, all the operations up to the stage of chromatography were performed in gloved boxes, through which a rapid draft of air was maintained by a suction fan, and the whole system was very carefully monitored to ensure that no activity escaped from the confines of the box and the venting system. A description of the apparatus in which the actual photosynthesis experiments were performed has been given earlier,[2] together with a description of the experimental procedure.

After extraction of the cells with ethanol, the extracts were four times evaporated to dryness and redissolved in ordinary water in order to wash out any exchangeable tritium present in the compounds extracted from the cells, leaving the tritium present only in nonexchangeable positions. The final residue was dissolved in water and chromatographed in the usual way, and the chromatograms were exposed to x-ray film to find the locations of the radioactive materials. The tritiated water was prepared as described by I. M. Whittemore and R. L. Lehman.[3]

---

[2] V. Moses, in Chemistry Division Quarterly Report, UCRL-8141, Dec. 1957, p. 17.

[3] I. M. Whittemore and R. L. Lehman, Synthesis and Storage of Small Quantities of Tritiated Water, UCRL-8056, Nov. 1957.

A necessary preliminary to these studies was to establish that the flux of radiation due to a specific activity of 1 C of tritium per ml for 3 minutes was not likely to damage the cells, or cause derangement of their photosynthetic capabilities. The radiation dosage for 3 min was about 760 rep. An experiment was performed in which equal aliquots of Chlorella cells were allowed to carry on photosynthesis in the presence of $C^{14}O_2$ for 3 min in the presence and absence of HTO. Chromatograms were prepared and exposed to x-ray film with a layer of Mylar placed between the paper and the film. The Mylar was sufficient to absorb the tritium radiation but not to affect very greatly that from the C^{14}. A number of compounds from each sample was assayed for radioactivity and the results are presented in Table I. These compounds normally contain 80 to 90% of the total C^{14} in cells supplied with $C^{14}O_2$ for 3 min in the light. Apart from the changes in glycolic acid and in glutamic acid and sucrose, no very significant alteration of the photosynthetic pattern resulted from the presence of tritiated water.

The substances incorporating tritium from water when the cells are suspended on phosphate buffer alone, buffer plus ammonium chloride, and buffer plus potassium nitrate, all in the light, and in phosphate buffer alone in the dark, are shown in Table II. The identities of all these substances have been confirmed by standard techniques of elution, treatment with acid phosphatase where appropriate, and cochromatography with authentic markers. Further kinetic studies are in progress to determine the sequence in which tritium appears in the various substances.

While cells incorporate C^{14} from $C^{14}O_2$ during photosynthesis for short periods mainly into sugar phosphates and into sucrose (these two groups of substances frequently account, after 3 min, for 75 to 85% of the total labeled substances present in the ethanol and water extracts of the cells), tritium is found in 3 min largely in glutamic, aspartic, and malic acids and alanine, which together contain about 63% of the soluble tritium fixed (excluding glycolic acid--see below), and none appears in sucrose. Most significant is the amount appearing in glycolic acid. This substance is quite volatile, yet even after the cell extract has been evaporated to dryness four times, glycolic acid remains the most radioactive spot on the chromatogram. It seems probable that the overwhelmingly greatest quantity of tracer is incorporated into glycolic acid. The significance of this is not yet known. Owing to its volatile nature, it was not possible to confirm the identity of the glycolic acid spot by cochromatography. However, the fact that it is volatile, together with its chromatographic behavior, leaves very little doubt as to its nature. Although larger amounts of tritium are incorporated in the light than in the dark, nevertheless considerable amounts are also incorporated in the absence of light (far greater, proportionately, than with carbon dioxide), and the identities of the compounds in which tritium appears in the light and the dark are similar.

Table I

Effect of high-specific-activity tritiated water on the incorporation of labeled carbon dioxide into several substances

Cells exposed for 3 min to $NaHC^{14}O_3$ (40 μC/ml) with or without HTO (1C/ml) Values are counts/min for the compounds in a standard aliquot.

	Substrate; and dose of radiation received per 3 min	
	$C^{14}O_2$	$C^{14}O_2$ plus HTO
	0.24 rep	760 rep
Sugar diphosphates	31,350	34,200
Sugar monophosphates	189,050	180,500
Phosphoglyceric acid	105,450	84,550
Uridinediphosphoglucose	26,100	21,400
Aspartic acid	13,525	7,700
Glutamic acid	3,230	2,100
Alanine	19,750	14,900
Malic acid	25,650	21,200
Fumaric acid	3,980	3,565
Glycolic acid	24,900	38,000
Sucrose	9,750	4,050
Total	452,735	412,165

Table II

Incorporation of tritiated water in the light and in the dark; effect of presence of ammonium chloride and potassium nitrate on the uptake of tritiated water in the light

Cells exposed for 3 min to 1 C of HTO, together with $C^{12}O_2$, in the light or dark, and in the media stated. Values are counts/min for the compounds in a standard aliquot.

	Medium; light conditions							
	Phosphate		Phosphate + NH_4Cl		Phosphate + KNO_3		Phosphate	
	Light		Light		Light		Dark	
	cpm	%	cpm	%	cpm	%	cpm	%
Sugar diphosphates	792	3.3	582	2.4	415	1.6	0	0.0
Sugar monophosphates	3866	16.1	3101	12.6	3095	12.3	1401	11.1
Phosphoglyceric and phosphoglycolic acids	1281	5.4	1250	5.1	1346	5.3	1065	8.4
Phosphoenolpyruvate[a]	123	0.5	87	0.4	90	0.4	0	0.0
Uridinediphospho-glucose	629	2.6	303	1.2	919	3.7	367	2.9
Fumarate	372	1.6	460	1.9	224	0.9	(b)	0.0
Malate	4412	18.4	5478	22.2	1953	7.8	454	3.6
Citrate	164	0.7	380	1.5	288	1.1	0	0.0
Succinate[a]	448	1.9	284	1.2	376	1.5	162	1.3
(Glycolate)[a]	(339)	(1.4)	(74)	(0.3)	(64)	(0.3)	(b)	(0.0)
Aspartate	1951	8.1	2317	9.4	3722	14.8	3347	26.5
Glutamate	7781	32.5	7353	29.8	7899	31.4	4194	33.2
Serine plus glycine[c]	900	3.8	653	2.6	537	2.1	138	1.1
Alanine	987	4.1	1753	7.1	3056	12.1	1518	12.0
Glutamine	187	0.8	328	1.3	817	3.2	0	0.0
Unknowns	48	0.2	316	1.3	441	1.7	0	0.0
Totals[d]	23,941		24,645		25,178		12,646	

[a] Identity not confirmed by cochromatography

[b] Trace of blackening on film

[c] Mainly serine

[d] Excluding glycolic acid

The interpretation of these results is likely to be a complicated matter. While the tritium may be incorporated by genuine biochemical reductions into many compounds under investigation, it is also possible that tritium may originally enter some substances by nonspecific exchange reactions, and, subsequently, be relocated into nonexchangeable positions. Further, tritium, incorporated by a genuine biochemical reduction into a nonexchangeable position, may later be removed to an exchangeable one and hence be lost in the repeated evaporations. If the latter are not performed, it is probable that every compound on the paper that contains exchangeable hydrogen atoms will show radioactivity, whether or not it had played any part in the metabolic reactions under examination. Finally, water may be taken up without first being split into hydrogen and oxygen, as in the reactions producing malic from fumaric acids, or isocitric from aconitic acids.

In spite of all these difficulties, it seems likely that the use of tritium will eventually enable us to learn something of the path of hydrogen, although not with the ease or completeness with which our studies with C^{14} have taught us the path of carbon in photosynthesis.

THE NONPHOTOSYNTHETIC FIXATION OF CARBON DIOXIDE BY THREE MICROORGANISMS

V. Moses and Osmund Holm-Hansen

A study is currently being made of the nonphotosynthetic pathways of carbon dioxide fixation in three microorganisms, two algae and a fungus. The algae were incubated for various periods with labeled sodium bicarbonate in total darkness, and were killed and extracted at the end of the desired incubation periods with boiling ethanol. The extracts were chromatographed by using standard procedures, and the various labeled compounds were assayed for activity. As the fungus is incapable of photosynthesis, no precautions to exclude light were necessary, but otherwise the techniques used were the same as those for the algae.

The two algae studied were Chlorella pyrenoidosa and Nostoc muscorum. The cells were suspended in distilled water at a cellular concentration of 2 to 3%. The cells were shaken in the dark in air for 30 min, following which period labeled bicarbonate was added and the cells were incubated for 19, 32, 46, 60, 121, 183, and 1800 sec in the case of Chlorella, and for 3, 10, 30, 60, 180, and 1800 sec with Nostoc. Tables III and IV show the patterns of fixation of $NaHC^{14}O_3$ into these two organisms in the dark.

The fungus Zygorrhynchus moelleri was grown in a glucose–ammonium chloride–mineral salts medium for about 18 to 20 hr, and after harvesting and washing, it was suspended in distilled water at a cell concentration of 5%. The cells were incubated with $NaHC^{14}O_3$ for periods of 4, 11, 15, 30, 45, 60, 75, 90, 105, 135, 150, 165, and 180 sec, and in a separate experiment for 1800 sec. The patterns of $C^{14}O_2$ uptake for some of these incubation periods are shown in Table V. A further experiment was performed in which the fungal cells

Table III

Fixation of $C^{14}O_2$ in the dark by Chlorella pyrenoidosa.

The values given are expressed as the percent of the total soluble C^{14} appearing in each compound.

	Incubation period (sec)						
	19	32	46	60	121	183	1800[a]
Sugar monophosphates							1.2
Triose phosphates[b]							0.6
Phosphoglyceric acid					0.6	0.4	0.5
Phosphoenol-pyruvic acid							0.1
Uridine-phosphate-sugar[b]							1.0
Glucose[b]							1.0
Fumarate		1.8	6.9	3.3	3.0	3.6	3.0
Succinate							1.7
Malate	27.5	22.3	19.4	16.3	13.3	14.3	15.7
Citrate	10.1	6.5	7.0	3.8	4.6	3.9	1.6
Aspartate	40.4	50.7	45.5	56.2	58.2	55.6	26.9
Glutamate			6.8	5.9	9.3	11.9	35.2
Alanine	21.9	18.6	14.5	14.6	11.0	10.4	2.0
Serine plus glycine							1.1
Unknowns							8.1
Total activity fixed (dpm/100 µl cells $\times 10^{-5}$)	0.089	0.245	0.377	0.596	0.822	1.68	12.10

[a] Separate experiment

[b] Identity not confirmed

Table IV

Fixation of $C^{14}O_2$ in the dark by Nostoc muscorum

The values given are expressed as the percent of the total soluble C^{14} appearing in each compound.

	Incubation period (sec)					
	3	10	30	60	180	1800
Sugar monophosphates					1.5	5.0
Pentose phosphates[a]					1.6	1.2
Phosphoglyceric acid					1.8	1.1
Phosphoenolpyruvic acid						0.2
Sucrose						2.2
Malate					0.9	0.3
Citrate				4.8	1.8	2.5
Aspartate	100.0	100.0	100.0	95.2	80.2	40.5
Glutamate					4.6	24.8
Alanine					1.4	2.3
Glutamine					1.3	1.7
Threonine						2.6
Citrulline					4.1	7.0
Unknowns					0.8	8.6
Total activity fixed (dpm/100 µl cells $\times 10^{-5}$)	0.023	0.008	0.034	0.165	1.59	4.50

[a]Identity not confirmed.

Table V

Fixation of $C^{14}O_2$ by Zygorrhynchus moelleri

The values given are expressed as the percent of the total soluble C^{14} appearing in each compound.

	Incubation period (sec)						
	4	11	30	60	105	180	1800[a]
Sugar monophosphates[b]							1.2
Phosphoglyceric acid							0.8
Phosphoenolpyruvic acid				0.6	0.2	0.02	0.3
Phosphogluconic acid	10.9	11.1	6.5	1.1	0.4	0.2	0.1
Uridine triphosphate					0.4	1.1	1.0
Uridinediphosphoribose			0.7	2.0	5.3	4.0	1.3
Uridinediphosphoglucose				0.1	1.2	1.8	1.3
ATP(probably plus UDP)			0.3	2.7	4.4	2.8	1.0
Combined nucleoside diphosphates[c]			0.5	0.7	3.1	4.9	21.5
Unidentified nucleoside monophosphate				0.8	1.0	0.8	
Maltose				0.8	0.5	0.3	0.2
Fructose					1.5	3.0	
Fumarate		6.0	1.0	2.3	1.4	1.5	0.9
Succinate							1.1
Malate	23.6	21.2	11.3	14.8	9.0	8.0	6.6
Citrate	65.5	39.0	31.0	11.2	7.6	5.2	2.4
Aspartate		22.6	40.9	51.5	48.9	49.5	19.9
Glutamate			2.2	3.3	4.4	5.1	20.5
Serine plus glycine				1.0	2.0	3.3	3.9
Threonine			2.2	0.7	1.3	2.3	2.3
Alanine			3.5	5.9	6.0	4.2	2.3
Proline					0.4	0.5	0.4
Valine				0.6	0.4	0.3	3.4
Glutamine					0.6	0.7	3.8
Citrulline							0.2
Unknowns						0.5	3.9
Total activity fixed (dpm/100 μl cells $\times 10^{-5}$)	0.0183	0.0965	0.336	1.48	4.43	8.12	12.8

[a] Separate experiment.

[b] Contains monophosphates of glucose, fructose and possibly sedoheptulose.

[c] Contains diphosphates of inosine, uridine, and guanosine.

were allowed to assimilate $NaHC^{14}O_3$ in the presence and absence of unlabeled glucose, a substance which these cells oxidize rapidly. There was an enhanced incorporation of $C^{14}O_2$ in the presence of unlabeled glucose (Table VI), but the patterns of activity were similar in appearance and were not analyzed further.

The results with both the algae indicate that carbon dioxide is fixed in the dark entirely through carboxylations associated with the tricarboxylic acid cycle. In both cases aspartic acid is very prominent during the shorter incubation periods, and, in fact, with Nostoc is the only labeled substance formed from $C^{14}O_2$ for as long as 30 sec. Later on other Krebs-cycle compounds appear. In Chlorella, activity appears in the sugar phosphates after 30 minutes. The evidence for the route by which activity enters the sugars favors the operation of a reversal of the glycolytic breakdown of these substances. Thus in Chlorella phosphoglyceric acid is the first sugar phosphate to become labeled, followed at a later stage by phosphoenolpyruvate, thriose phosphate, and the hexose monophosphates. In Nostoc activity can be observed in phosphoglyceric acid, and pentose and hexose phosphates after 3 min. A significant amount of activity in this organism is incorporated into citrulline, an observation first made by Linko, Holm-Hansen, Bassham, and Calvin,[1] though no citrulline was seen in Chlorella under these conditions.

The results obtained with the fungal cells are somewhat different. In these cells activity appeared in the shortest periods in malic, citric, and phosphogluconic acids (4 sec). Immediately after this, fumaric and aspartic acids became labeled (11 sec), and the isotope became ever more widespread in substances associated with the citric acid cycle after 30 sec, by which time glutamic acid, threonine, and alanine also contained activity. Considerable activity was also incorporated into the nucleotides (Table V). However, although phosphogluconate contained C^{14} after the shortest incubation period, and very small amounts of activity began to be observable in phosphoenolpyruvate after 45 sec, phosphoglycerate and the hexose monophosphates did not become labeled until some time between 3 and 30 min, and at no time was activity seen in the sugar diphosphates. This was true even when these cells assimilated $C^{14}O_2$ while simultaneously oxidizing unlabeled glucose. It is known from other studies that this organism metabolizes glucose at least partly via fructose diphosphate, so that a metabolic pool of this substance must have been present.[2] It would appear, therefore, that, as well as being incorporated into Krebs-cycle intermediates by carboxylation of pyruvic acid, C^{14} from $C^{14}O_2$ entered the hexose phosphate by a route involving carboxylation of a pentose phosphate to give phosphogluconate (the first labeled sugar phosphate observed), which was then reduced to hexose phosphate.

These findings appear particularly significant with regard to the questions of the physical separation within the cells of different metabolic pools of the same substance. Glucose is known to be metabolized in these cells via fructose diphosphate (and glycolysis), and also by the decarboxylation of phosphogluconate to yield pentose phosphate. Yet when the cells are supplied with labeled carbon dioxide, activity appears first in phosphogluconate, and

---

[1] Linko, Holm-Hansen, Bassham, and Calvin, J. Exptl. Botany 8, 147 (1957).

[2] V. Moses, in Chemistry Division Quarterly Report, UCRL-3950, Sept. 1957, p. 30.

Table VI

Fixation of $C^{14}O_2$ by Zygorrhynchus moelleri in the presence and absence of glucose

The values are given in counts $\times 10^{-3}$/min for a standard aliquot.

	Incubation period (sec.)		
	45	180	1800
With glucose	121	421	1083
No glucose	54.7	263	830

is present later in the hexose monophosphates although absent from the hexose diphosphates. This would seem to indicate firstly that $C^{14}O_2$ is assimilated into hexose by a pentose carboxylation and not by a reversal of glycolysis, and secondly that there must be two pools, at least of phosphogluconate and of hexose monophosphates, to account for the absence of label in the hexose diphosphates. Other cases are known in biology of such a physical separation of metabolites, one well-known example being the accumulation of isocitrate by the plant Bryophyllum. The enzyme aconitase catalyzes the conversion of citric acid to isocitric acid, the equilibrium state being about 94% citrate and 6% isocitrate. Aconitase isolated from Bryophyllum appears to be normal, yet the plant is able to accumulate isocitric acid in the vacuoles. Presumably this can only be done by preventing the isocitrate from coming into contact with the aconitase, and thus maintaining the stored isocitrate from the metabolically active pool of this acid which participates in the tricarboxylic acid cycle.

THE TENTATIVE IDENTIFICATION OF ERYTHROSE PHOSPHATE

V. Moses

In the course of the elucidation of the photosynthetic carbon cycle it was necessary to propose a role for erythrose-4-phosphate as an intermediate in the series of reactions resulting in the formation of sedoheptulose phosphate(s). It was postulated that 3-phosphoglyceraldehyde and fructose-6-phosphate underwent a transketolase reaction to form xylulose-5-phosphate and erythrose-4-phosphate. The latter would then combine with dihydroxyacetone phosphate under the influence of aldolase to yield sedoheptulose-1, 7-diphosphate,[1] or—under the influence of transaldolase with fructose-6-phosphate—to form sedoheptulose-7-phosphate.[2]

Although both the formation of erythrose-4-phosphate from 3-phosphoglyceraldehyde and fructose-6-phosphate, and its combination with dihydroxyacetone phosphate to form sedoheptulose-1, 7-diphosphate have been demonstrated in vitro,[3, 4, 5] erythrose-4-phosphate has not, until now, been detected in extracts of algae allowed to carry on photosynthesis in the presence of $C^{14}O_2$. In addition, as far as we are aware, erythrose-4-phosphate has not been found to occur naturally in any other plant system. It was therefore of interest to attempt to isolate the tetrose phosphate from extracts of algae supplied with labeled carbon dioxide. The xylulose-5-phosphate formed by the transketolase reaction has already been reported in algae.[6]

Chlorella pyrenoidosa was incubated in the light for 3 minutes in the presence of $C^{14}O_2$. The cells were killed with ethanol and extracted, the extract was chromatographed as described by Moses and Calvin[7] to achieve a good separation of the substances lying in the area near the origin which contains the sugar phosphates, and a radioautogram was obtained by exposure of the chromatogram to x-ray film.[8] Fourteen or more spots were present in the area containing the triose and pentose phosphates, lying between the hexose monophosphates and free sucrose (Fig. 1). Each of these, and also the hexose monophosphate area itself, was eluted, treated with human seminal acid phosphatase in acetate buffer, pH5, and rechromatographed with an authentic sample of erythrose. The erythrose was prepared from

---

[1] Bassham, Benson, Kay, Harris, Wilson, and Calvin, J. Am. Chem. Soc. 76, 1760 (1954).

[2] E. Racker, Nature 175, 249 (1955).

[3] Horecker, Gibbs, Klenow, and Smyrniotis, J. Biol. Chem. 207, 393 (1954).

[4] Horecker, Smyrniotis, Hiatt, and Marks, J. Biol. Chem. 212, 827 (1955).

[5] Racker, de la Haba, and Leder, Arch. Biochem. Biophys. 48, 238 (1954).

[6] Bassham, Barker, Calvin, and Quarck, Biochim. et Biophys. Acta 21, 376(1956).

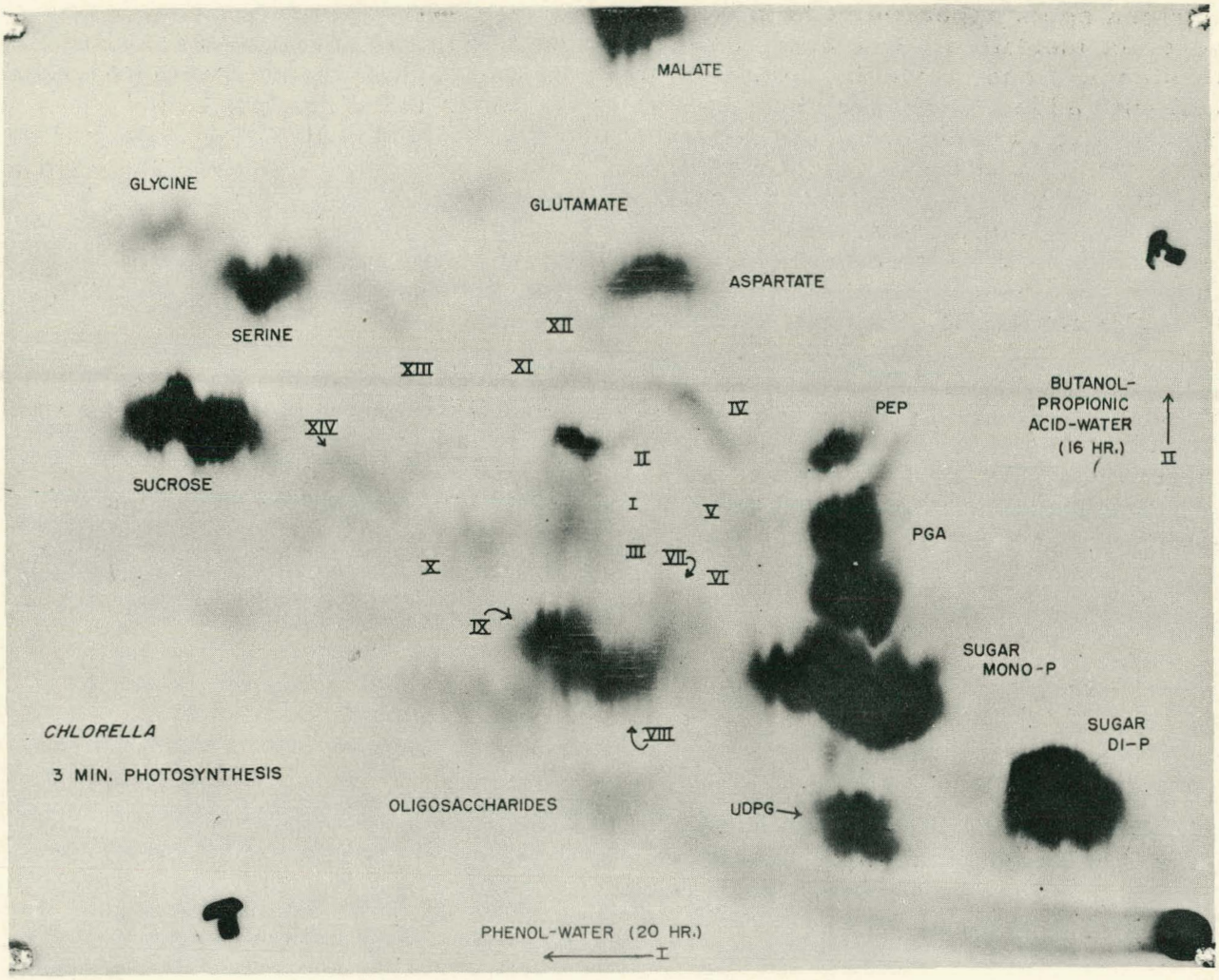
[7] V. Moses and M. Calvin, Proc. Natl. Acad. Sci. U.S. 44, 260 (1958).

[8] Benson, Bassham, Calvin, Goodale, Haas, and Stepka, J. Am. Chem. Soc. 72, 1710 (1950).

4,6-ethylidene glucose by periodate oxidation, followed by hydrolysis of the 2,4-ethylidene erythrose and the purification of the free tetrose.[9] All the fourteen spots appeared to be mixtures of several phosphates, most of which were not identified. One spot, however (Spot I, Fig. 1), contained several substances, one of which, after treatment with phosphatase, cochromatographed well with added erythrose, but not with erythrulose (Fig. 2). This spot (Spot I, Fig. 1) also showed other substances after phosphatasing: glycolic and glyceric acids were identified (Spots XV and XVI, Fig. 2) and a substance present in only the minutest traces (Spot XVIII, Fig. 2) had the same chromatographic parameters as erythronic acid; the amount present was much too small to attempt cochromatography and "finger-printing" with authentic erythronic acid. The labeled erythronic acid probably arose from air oxidation of the erythrose on the paper.

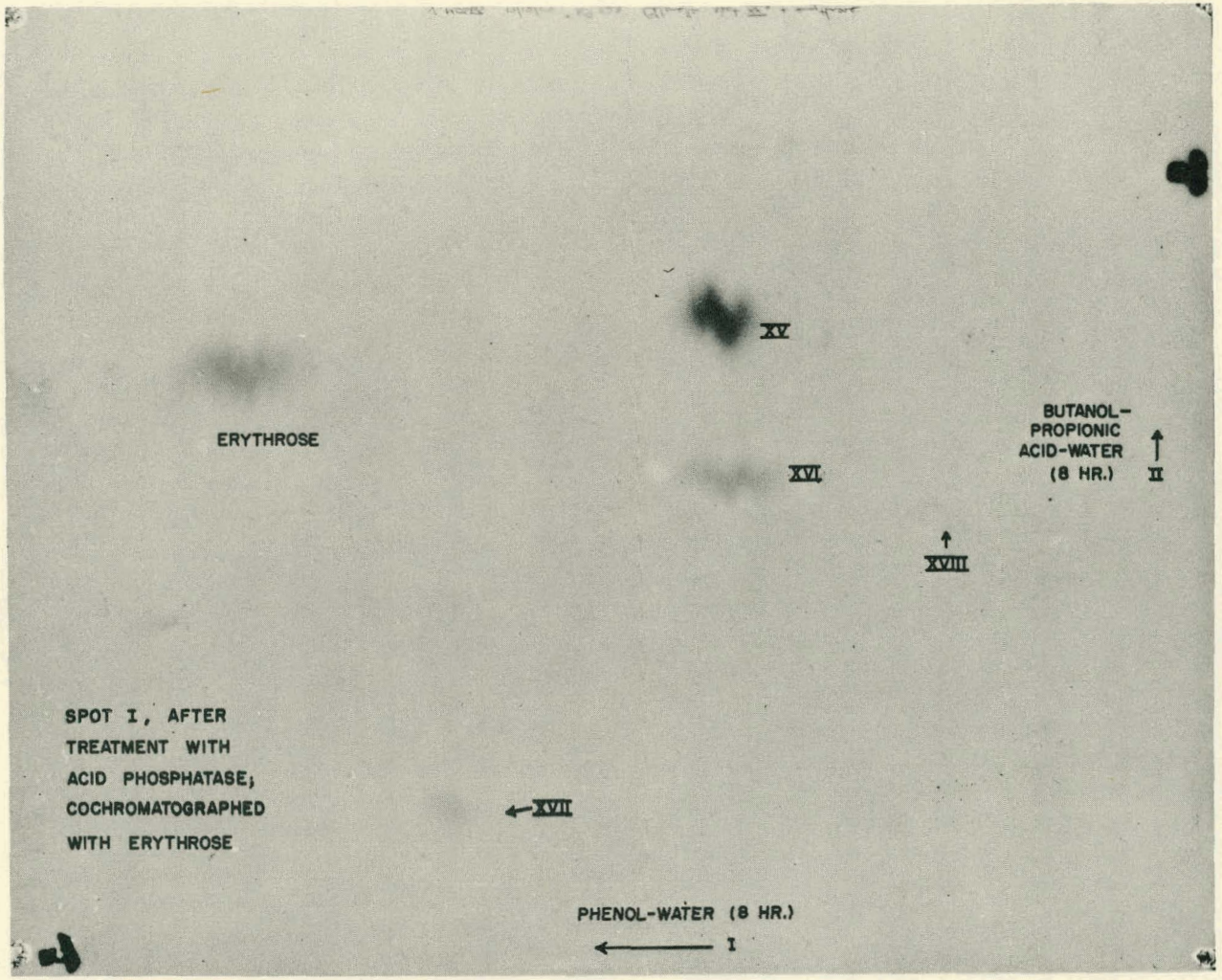
Treatment of the hexose monophosphates with phosphatase also produced a substance that cochromatographed with erythrose. Whether or not this indicates that two forms of erythrose phosphate were present in the cell extract, or whether that derived from the hexose monophosphate area resulted from a decomposition of some other substance in that area, is not known.

[9] D. A. Rapoport and W. Z. Hassid, J. Am. Chem. Soc. 73, 5524 (1951).



ZN-2021

Fig. 1. Radioautogram of Chlorella extract after 3 minutes' photosynthesis with $C^{14}O_2$, chromatographed to separate the sugar phosphates. After treatment with acid phosphatase, the following substances were tentatively identified from the numbered spots: erythrose; glycolic, glyceric, and erythronic acids from I; glycolic, glyceric, and erythronic acids from II; dihydroxyacetone and other substances from III. Spot IV appeared to contain pentose phosphate, spot IX glucose-1, 2-cyclic phosphate, and spot X possibly a phosphate ester of a sugar alcohol.



ZN-2022

Fig. 2. Spot I, from Fig. 1, after treatment with acid phosphatase. Identities of the numbered spots: XV, glycolic acid; XVI, glyceric acid; XVII, not identified; XVIII, probably erythronic acid.

STEADY-STATE STUDIES OF THE CARBON-REDUCTION CYCLE IN PHOTOSYNTHESIS

James A. Bassham and Martha R. Kirk

Previous studies of the steady state during photosynthesis by algae with $C^{14}O_2$ have provided valuable information regarding the details of the biochemical transformations which occur in the carbon-reduction cycle.[1,2] It has proved possible to measure the concentration of intermediates in the cycle during such a period of steady-state conditions by allowing algae, either Chlorella or Scenedesmus, to photosynthesize with $C^{14}O_2$ of constant specific activity for periods in excess of 20 minutes. By this time the intermediates in the cycle are "saturated" with respect to radiocarbon. In other words, the specific activity of each carbon atom of each intermediate is the same as the specific activity of the entering carbon dioxide. Consequently, if a sample of algae is taken under these conditions, killed in alcohol, extracted, and analyzed by the usual methods of paper chromatography and radioautography, and if the total radioactivity per unit weight of algae is determined, it becomes possible to calculate the concentration of each intermediate of the cycle in the active metabolic pool. At the same time, the rate of photosynthesis can be determined by means of instruments which monitor continuously the levels of C^{14}, CO_2, and oxygen in the gas which circulates in a closed system through the suspension of algae.[2]

With these techniques available, one can study the concentration of intermediates in a dynamic biochemical system as a function of the over-all rate of metabolism. In this instance the over-all rate of metabolism may be controlled conveniently by control of the incident light intensity on the algae. Rates of photosynthesis varying by a factor of five may be easily achieved.

Such a study has now been undertaken. However, several practical problems, which have been neglected in the semiquantitative studies previously performed, had to be overcome. First of all, a more careful temperature control was required, and this has been achieved by placing a water jacket on either side of the algae illumination cell and rapidly circulating water from a thermostated bath through this jacket. Secondly, it was found in a preliminary experiment that during the course of the experiment the pH of the algae suspension increased over 1.5 pH units when the nutrient solution and density of algae used in earlier experiments were employed. Aside from possible changes in the steady state which might accompany such a pH change, this change resulted in a substantial change in the effective volume of the system for carbon dioxide, owing to the increased concentrations of bicarbonate at the higher pH's. Therefore, pH electrodes have been installed in the cell and steps have been taken to control the pH within narrower limits by adjusting the concentration of inorganic salts in the nutrient and also the concentration of the algae. It was also found that the algae grew so rapidly during the course of the experiment, which lasted about 2 hours, that the density of

---

[1] A. T. Wilson and M. Calvin, J. Am. Chem. Soc. 77, 5948 (1955).

[2] Bassham, Shibata, Steenberg, Bourdon, and Calvin, J. Am. Chem. Soc. 78, 4120 (1956).

algae was nearly 50% greater at the end of the experiment than at the beginning. This growth, of course, results in an apparent increase in the rate of photosynthesis, and inaccuracies in the measurements of the concentration. It is not possible to reduce the time of the experiment much below 90 minutes, since the algae should be allowed at least 20 minutes of any given set of conditions to reach a steady state. In addition, it is desirable to determine at least two different steady states for the sake of comparison in any given experiment, and also to repeat the first set of conditions after the second set as a check. Therefore, in order to adjust the concentration of algae during the course of the experiment an extra reservoir of nutrient solution was installed in the system, with provision for its addition to the algae suspension from time to time. In order to measure continuously the density of the algae suspension, a sample of the suspension is circulated past a small light and photoelectric cell, and as the algae grow the change in density of the suspension is indicated by means of the reading of the photoelectric cell. From time to time, fresh nutrient solution is added to bring the density of the algae back to the starting point. In order that the nutrient solution have the same bicarbonate concentration as the medium in which the algae are suspended, the stream of gas passes first through the algae suspension and then through the reservoir of nutrient solution.

Another problem has been to supply enough of critical inorganic salts, particularly nitrate, to insure that the concentration of these nutrients will not drop to levels at which they might limit the rates of some of the biochemical transformations during the course of the experiment. At the same time it has been necessary to hold the concentrations of salts at the minimum necessary level in order that the chromatographic analysis of the products of $C^{14}O_2$ fixation not be disturbed by salt effects. This problem has been partially solved by analyzing smaller aliquot samples of algae suspension and compensating for the smaller samples by employing $C^{14}O_2$ of higher specific activity.

The present conditions being employed in the studies are as follows:

1. Nutrient Solution:

KNO_3	1.5 g/l
$MgSO_4 \cdot 7H_2O$	0.2 g/l
KH_2PO_4	0.1 g/l
$K_2H\,PO_4 \cdot 3H_2O$	0.15 g/l
Trace elements (Hoaglund)	1 ml/l

2. Gaseous System:
   (a) Reservoir size: Large system - 5850 cc at pH 6.7; small system - 473 cc at pH 6.7.
   (b) Carbon dioxide concentration - 2.0% at the beginning of the experiment.
   (c) Total C^{14} activity - about 8 mC.
   (d) Volume of algae suspension - 50 cc.

(e) Density of suspension - 0.5 cc wet packed algae per 100 cc of suspension.

(f) Reservoir of nutrient solution at start of experiment - 30 cc.

(g) The size of the aliquot sample of algae suspension taken for chromatographic analysis -- 1 ml, which after killing and extraction and concentration, is divided into two equal portions and used for two chromatograms, one of which is run 16 to 20 hours in each solvent to achieve separation of the phosphate esters.

The results of experiments performed under the above conditions await development of chromatograms and radioautographs. Conditions will doubtless be changed when the results of this experiment are known.

BIOSYNTHETIC STUDIES ON MORPHINE ALKALOIDS

Henry Rapoport and Don Baker

A plant chamber for small-scale one-pot growth studies has been previously described.[1] In this chamber, Papaver somniferum (opium poppy) plants are being cultivated in the presence of radioactive carbon dioxide for the purpose of studying the path of carbon to the morphine alkaloids.

The plants were approximately the same age and had been grown under the same conditions prior to being placed in the plant chamber. A total of 1 mC of $C^{14}O_2$ was completely taken up by each plant in this series of studies. Plant No. 1 was exposed to radiocarbon for a 2-hour period and then harvested. Plant No. 2 was exposed to radiocarbon for 6 hours and then placed in the greenhouse in a special chamber for radioactive plants. After 5 days this plant was harvested. Plant No. 3 was exposed to radiocarbon for 6 hours and then placed in the special chamber for 10 days before it was harvested.

The morphine alkaloids were each separately isolated from the above plants and the specific activity of each alkaloid determined by procedures which will be described in a future report. The results are summarized in Table VII.

Experiments are now being done to determine the amount of radioactivity found in the methyl groups of morphine, codeine, and thebaine.

---

[1] Henry Rapoport, Don Baker, and Melvin Look, in Chemistry Division Quarterly Report, UCRL-8204, March 1958, p. 21.

Table VII

Specific activities of the isolated morphine alkaloids (disintegrations per minute per mg)

	Plant No. 1	Plant No. 2	Plant No. 3
Morphine	9,200	450,000	380,000
Codeine	21,000	705,000	424,000
Thebaine	75,000	670,000	> 600,000

SYNTHESIS OF 2-C-HYDROXYMETHYL PENTONIC ACIDS

Jan Anderson

In an investigation of the action of high concentrations of cyanide on the CO_2 fixation of green algae, Chlorella pyrenoidosa were treated with radioactive KCN.[1] One of the products appearing in these experiments was identified as hamamelonic acid diphosphate. This material was synthesized by the nonenzymatic cyanohydrin addition reaction on ribulose-1,5-diphosphate and shown (by cochromatography and electrophoresis) to be identical with the in vivo product.[2] Dephosphorylation of both the in vivo and the in vitro diphosphates yielded a hydroxy acid (or acids) closely related to hamamelonic acid (2-C-hydroxymethyl ribonic acid).

Furthermore, in the carboxylation of ribulose-1:5-diphosphate to form 3-phosphoglyceric acid, the β-keto acid postulated as an intermediate by Calvin[3] was identified by Moses.[4] Reduction of this β-keto acid with KBH_4 gave a hydroxy acid related to hamamelonic acid or its isomer.

In order to obtain the exact stereochemistry of the above acid or acids, it is necessary to have authentic samples of the four possible 2-C-hydroxymethyl pentonic acids.

---

[1] Joan M. Anderson and Duncan F. Shaw, in Chemistry Division Quarterly Report, UCRL-3950, Sept. 1957, p. 63.

[2] Brian R. Rabin, Duncan F. Shaw, Ning G. Pon, and Joan Mary Anderson, in Chemistry Division Quarterly Report, UCRL-3950, Sept. 1957, p. 23.

[3] M. Calvin, J. Chem. Soc. 1895 (1956).

[4] V. Moses, in Chemistry Division Quarterly Report, UCRL-8141, Dec. 1957, p. 11.

The method employed for the synthesis of the 2-C-hydroxymethyl pentonic acids consisted of the epimerization of the aldose (D-arabinose or D-xylose) to the corresponding ketose (ribulose or xylulose) by treatment with boiling pyridine. Separation of the ketose from the unreacted aldose was achieved by crystallization of their substituted phenylhydrazones. The ketose was then treated with cyanide following the method of Schmidt,[5] and attempts were made to separate the resulting epimeric acids.

Preparation of ribulose

D-arabinose (20 g) was refluxed gently for 4 hours in freshly distilled pyridine with all moisture excluded. The reaction mixture was evaporated under reduced pressure, and distilled water added and re-evaporated under reduced pressure; this was repeated several times until all traces of pyridine were removed. The resulting syrup was dissolved in absolute EtOH (40 ml) and allowed to cool, and some crystals of arabinose were added. The mixture was left for several days, during which time some of the unreacted arabinose crystallized out. After filtration, the syrup was evaporated under reduced pressure, dissolved in absolute EtOH (30 ml), and left for three more days. Yield of recovered arabinose: 14 to 16 g. The concentrated mother liquors yielded a brown syrup which consisted of about 70% to 80% ketose, as determined by optical rotation.

Several methods are available for the separation of the aldose from the ketose; the most satisfactory proved to be the synthesis of the benzylphenylhydrazone of the aldose (ketoses do not react with this reagent).

N-benzyl-N-phenylhydrazine hydrochloride (6.3 g) was dissolved in water (15 ml), treated with sodium acetate (2.5 g), and added to the syrup (5 g) obtained above. After gentle heating on a water bath for 3 hours the mixture was left overnight, partially concentrated under reduced pressure, and left until the yellow crystals of D-arabinose benzylphenylhydrazone (mp 174°) separated (8.3 g). The filtrate was evaporated under reduced pressure, dissolved in water (150 ml) to which benzaldehyde (3.2 ml) was added, and refluxed for 30 minutes. After cooling, the mixture was filtered as an oil; the aqueous layer was extracted several times with ether to remove any remaining benzaldehyde and evaporated under reduced pressure to give a yellow syrup which was dissolved in 80% EtOH, decolorized with charcoal, and evaporated again. This syrup corresponded to 100% ribulose as determined by chromatography, optical rotation, and the Bial orcinol test.[6]

Preparation of 2-C-hydroxymethyl pentonic acids

Following Schmidt's method,[5] the ketose was reacted with a slight excess of KCN (the pH adjusted to 5) and the reaction mixture left for 3 days in the cold room. After evaporation under reduced pressure to remove any excess HCN, hydrolysis of the cyanohydrins with $Ba(OH)_2$ was carried out at 60° for 2 hours; the reaction mixture was then evaporated under reduced pressure to remove NH_3. The barium salt was then treated with H_2SO_4 and

[5] O. T. Schmidt and K. Heintz, Ann. 515, 77 (1934).

[6] S. S. Cohen, J. Biol. Chem. 201, 71 (1953).

The free acid lactone mixture precipitated with $Ca(OH)_2$; the calcium salt was then treated with an equivalent amount of oxalic acid and an attempt was made to separate the epimeric acids by fractional crystallization of their phenylhydrazides. This method has several disadvantages: a considerable amount of material is lost in the purification of the various salts, since the acids are in equilibrium with their γ lactones at the pH's used, and furthermore, the separation of the phenylhydrazides appears somewhat tenuous.

A more satisfactory method is achieved if the reaction mixture after hydrolysis is run through an ion-exchange column of Dowex-50 at pH 2, thus separating the γ lactones from inorganic cations.

The actual separation of the acids has not yet been achieved; further work is being carried out with ion-exchange columns and fractional crystallization of the brucine salts.

THE RESPIRATORY METABOLISM OF LABELED COMPOUNDS BY DEUTERATED MICE

Ann M. Hughes

The effect of D_2O on the metabolism of several labeled compounds by mice has been studied.

C_{57} mice, both male and female, were maintained on 30% D_2O in the drinking water for 4 days or 2, 4, or 7 weeks before the metabolic studies were started. By use of the breath-line apparatus developed in this laboratory,[1] the respiratory metabolism of sodium acetate-2-C^{14}, glycine-2-C^{14}, formate-C^{14}, or glucose-U-C^{14} was followed for 4 hours. The dose of each labeled compound, injected intraperitoneally, was 1 mg. The amount of activity injected was about 10 μC for glycine, about 5 μC for all other compounds.

Results are shown in Figs. 3 to 14. There was no significant difference in the metabolism of acetate, glucose, or formate between the deuterated mice and controls maintained on regular water (Figs. 9 to 14). In the first series of experiments involving the metabolism of glycine, there was a marked difference between the deuterated and control mice after 2 weeks of treatment. The difference decreased with increasing length of treatment (Figs. 3 and 4). However, in two more series of animals, treated for 2 weeks, the large differences obtained in the first series were not reproduced (Figs. 5 and 6). Animals treated for only 4 days also showed no marked effect (Figs. 7 and 8).

[1] B. M. Tolbert, A. M. Hughes, M. R. Kirk and M. Calvin, Arch. Biochem. Biophys., 60, 301 (1956).

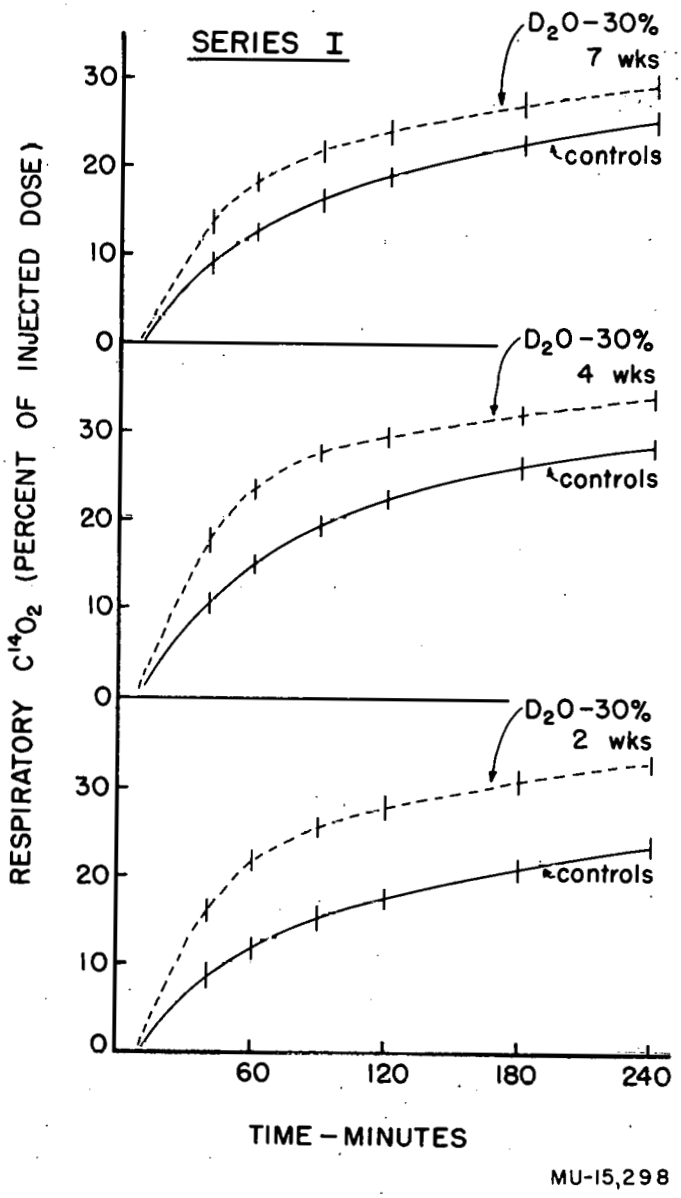


Fig. 3. Cumulative respiratory excretion of $C^{14}O_2$ by C_{57} mice after intraperitoneal injection of glycine-2-C^{14}. The standard error of the mean is indicated by a short vertical line for each point.*

*Standard error of the mean = $\sqrt{\frac{\Sigma(x-x^1)^2}{n(n-1)}}$.

After M. Calvin et al., Isotopic Carbon, (Wiley, New York, 1949).

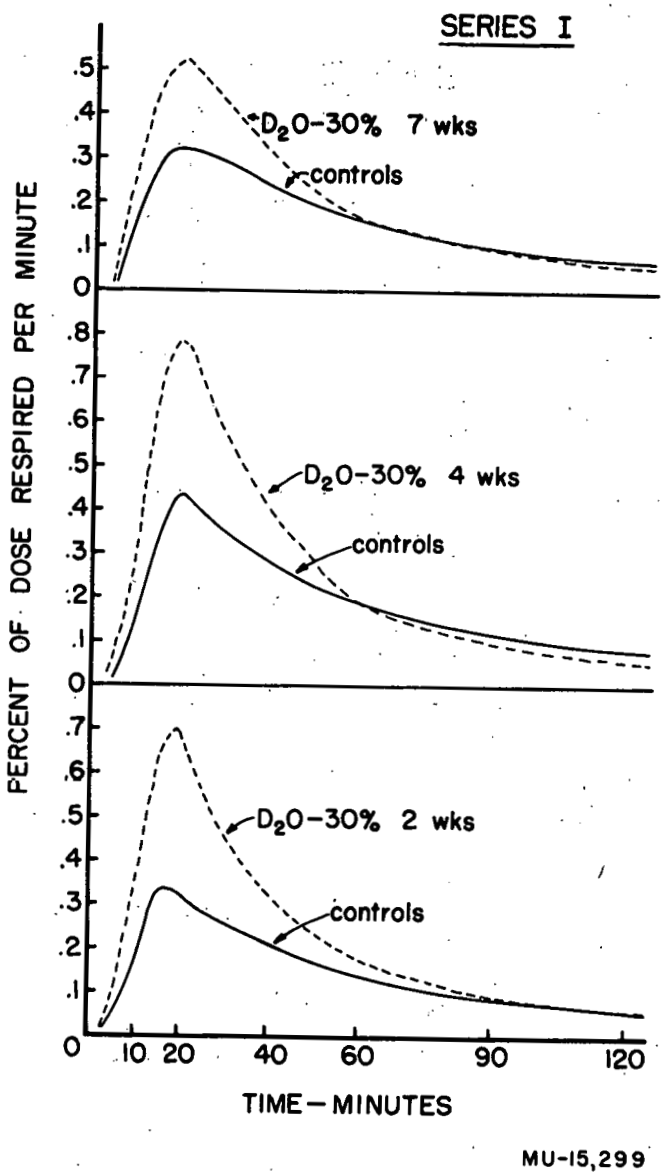


Fig. 4. Rate of respiration of $C^{14}O_2$ by C_{57} mice after intraperitoneal injection of glycine-2-C^{14}.

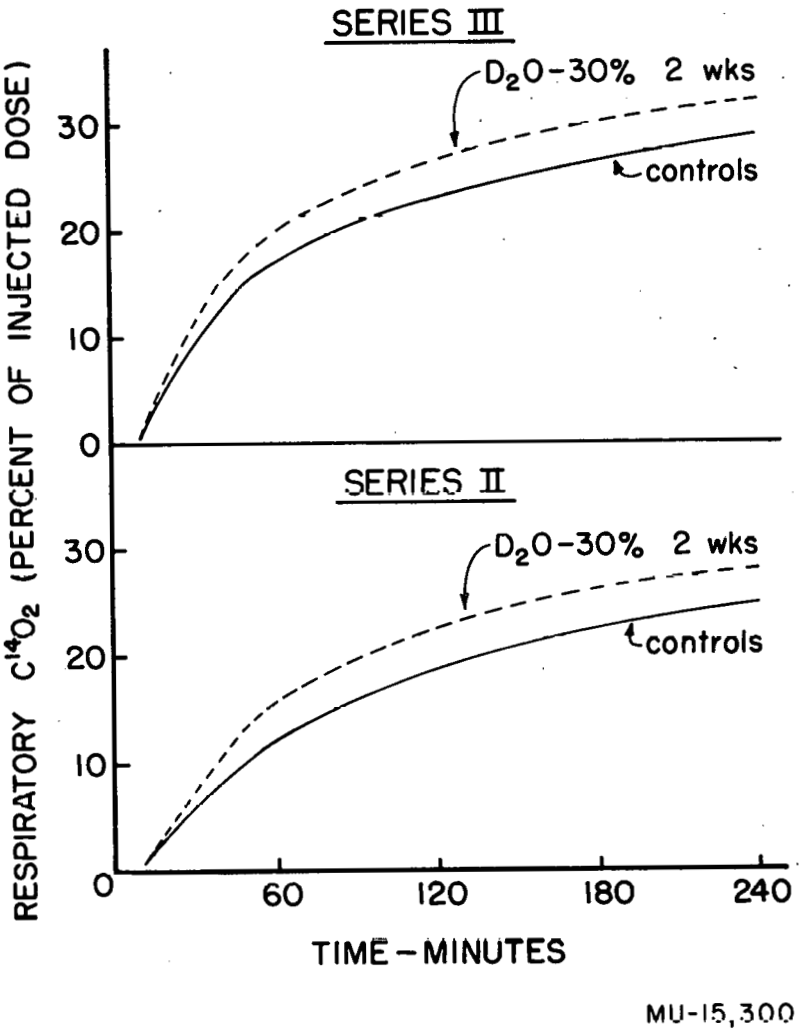


Fig. 5. Cumulative respiratory excretion of $C^{14}O_2$ by C_{57} mice after intraperitoneal injection of glycine-2-C^{14}.

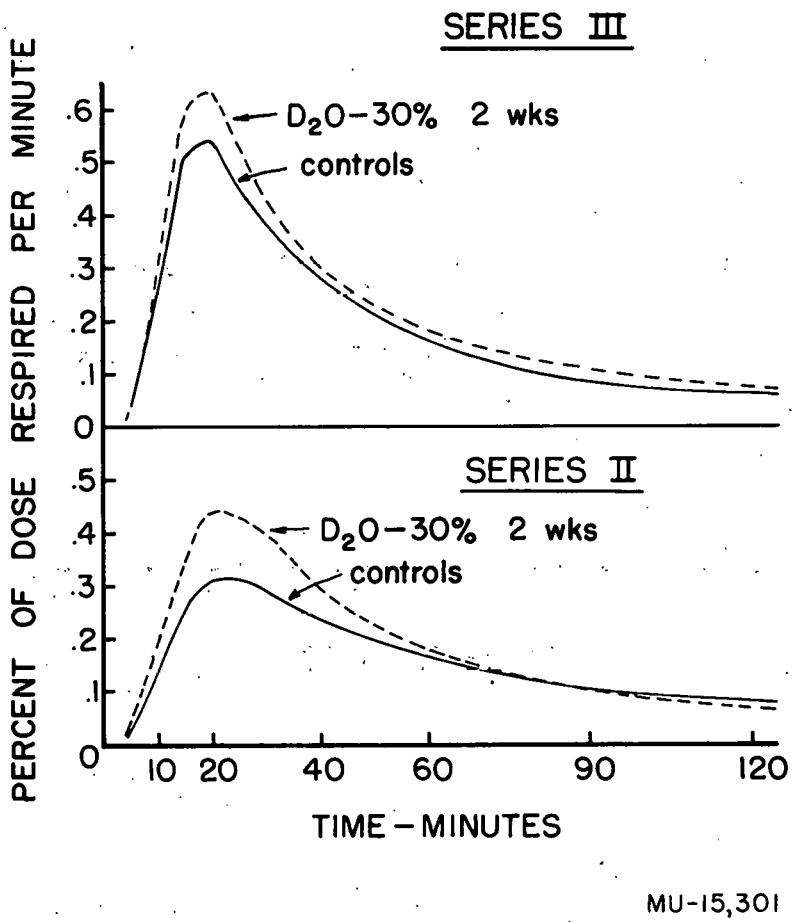


Fig. 6. Rate of respiration of $C^{14}O_2$ by C_{57} mice after intraperitoneal injection of glycine-2-C^{14}.

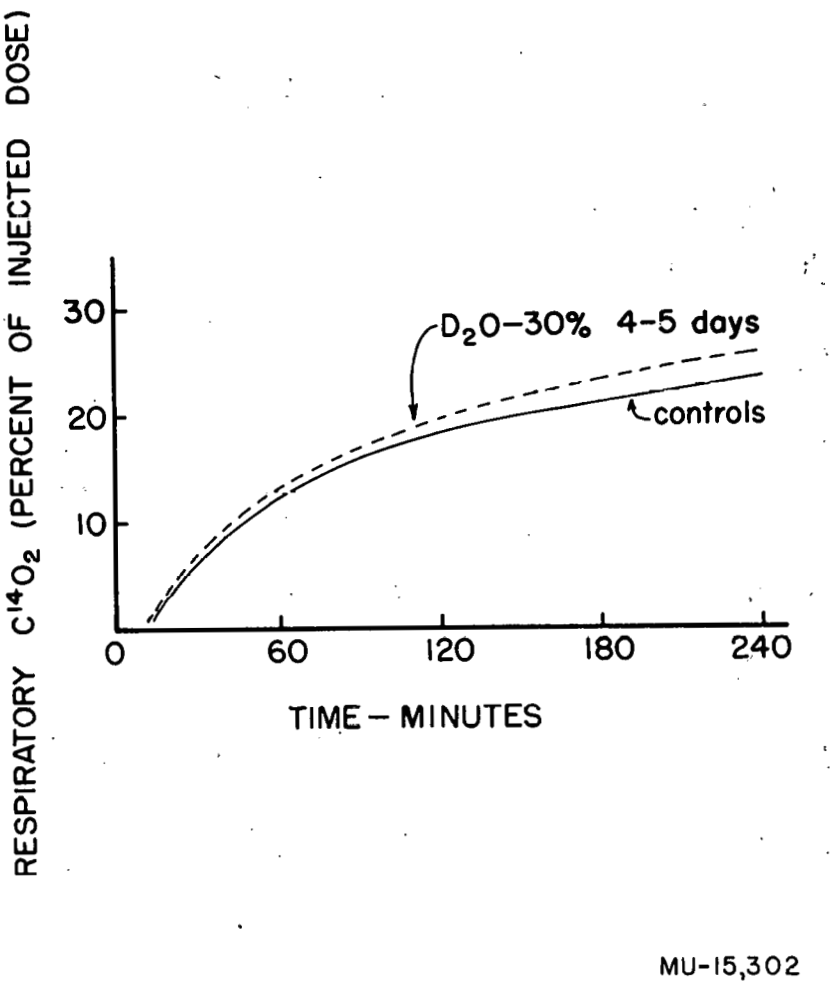


Fig. 7. Cumulative respiratory excretion of $C^{14}O_2$ by C_{57} mice after intraperitoneal injection of glycine-2-C^{14}.

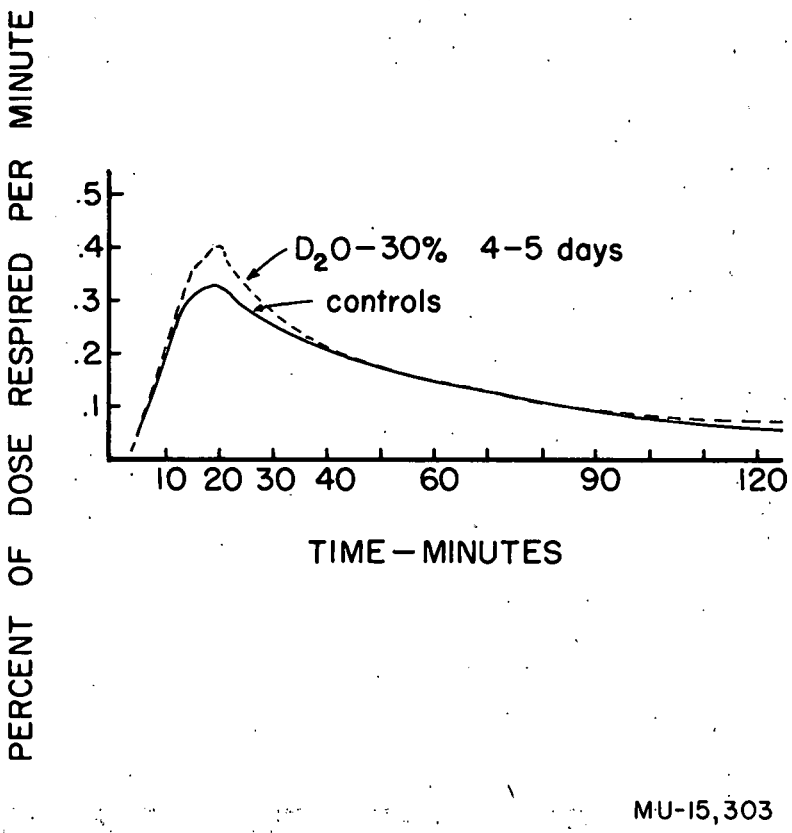


Fig. 8. Rate of respiration of $C^{14}O_2$ by C_{57} mice after intraperitoneal injection of glycine-2-C^{14}.

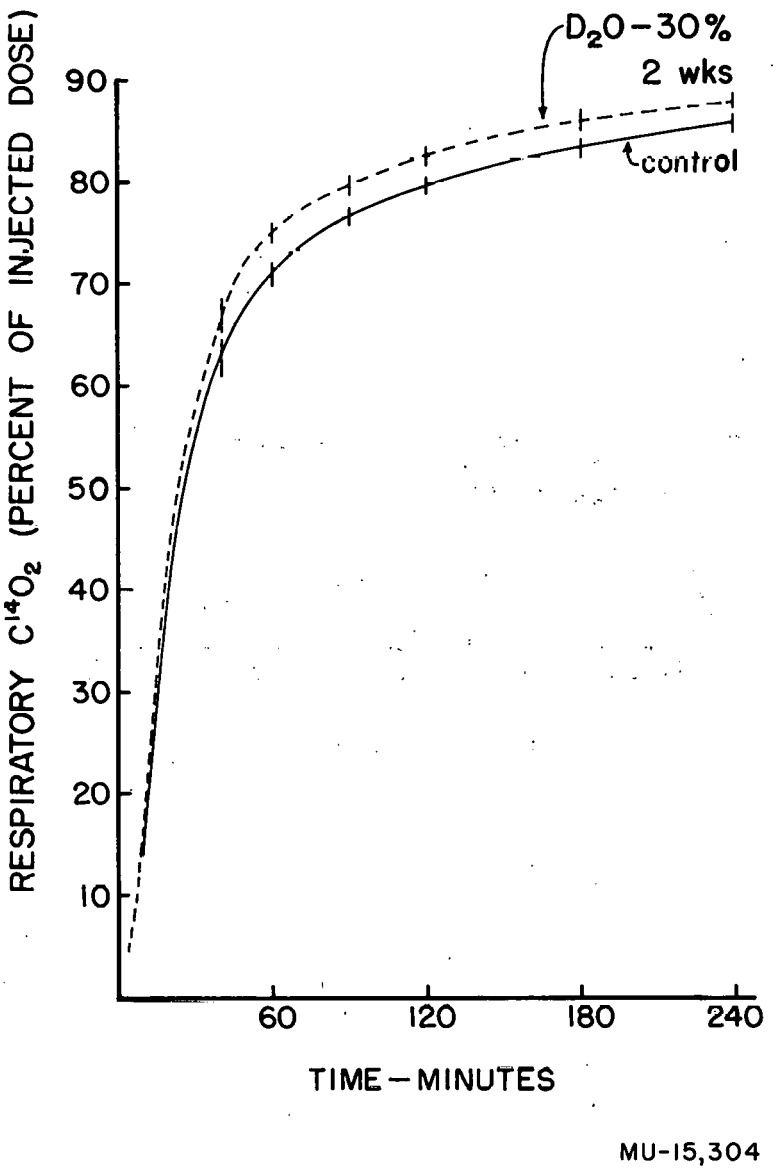


Fig. 9. Cumulative respiratory excretion of $C^{14}O_2$ by C_{57} mice after intraperitoneal injection of acetate-2-C^{14}.

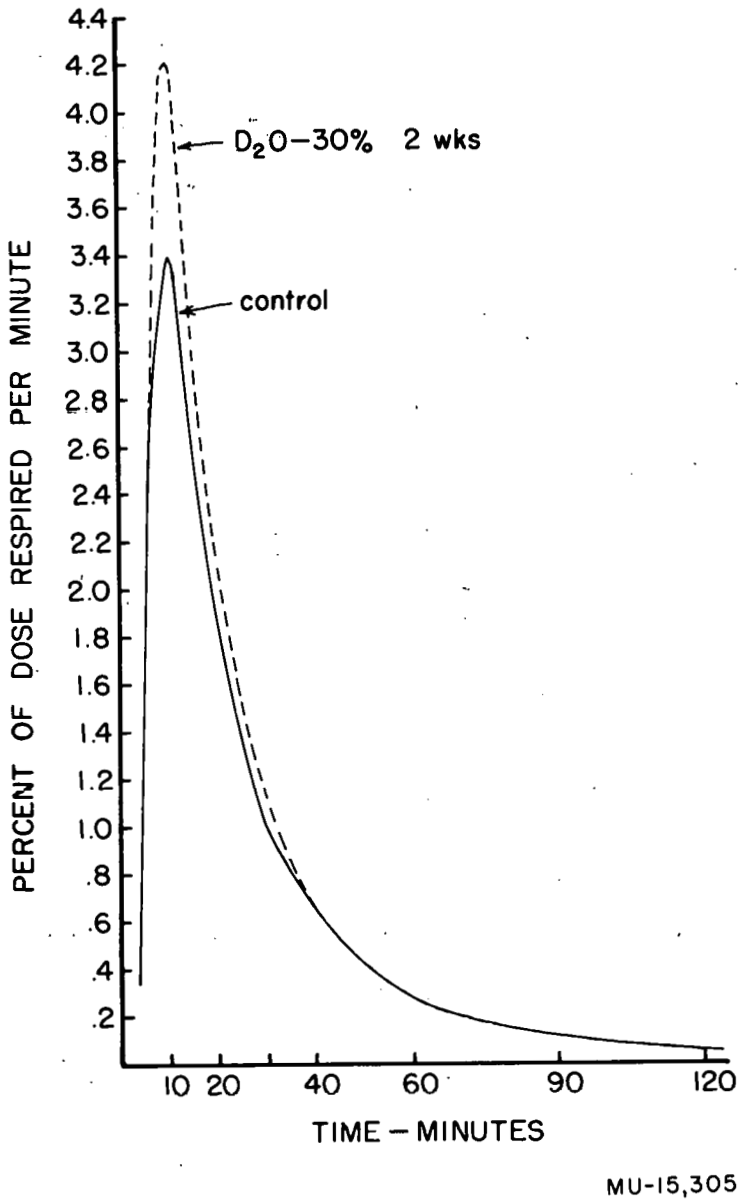


Fig. 10. Rate of respiration of $C^{14}O_2$ by C_{57} mice after intraperitoneal injection of acetate-2-C^{14}.

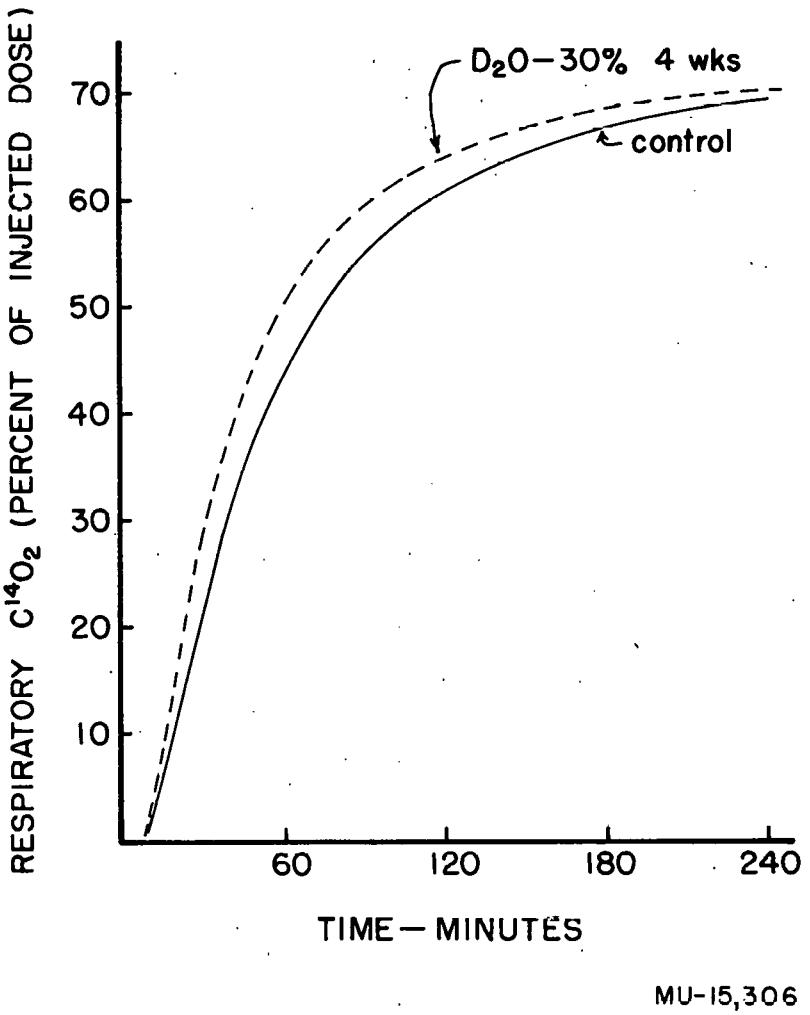


Fig. 11. Cumulative respiratory excretion of $C^{14}O_2$ by C_{57} mice after intraperitoneal injection of glucose-U-C^{14}.

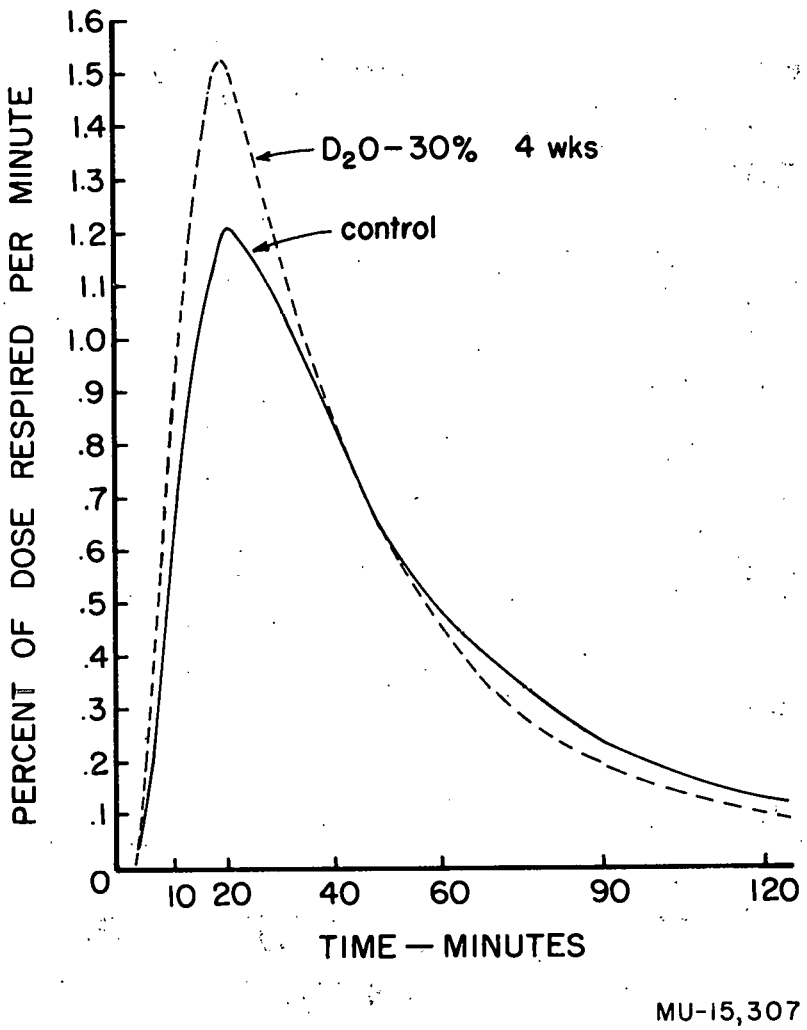


Fig. 12. Rate of respiration of $C^{14}O_2$ by C_{57} mice after intraperitoneal injection of glucose-U-C^{14}.

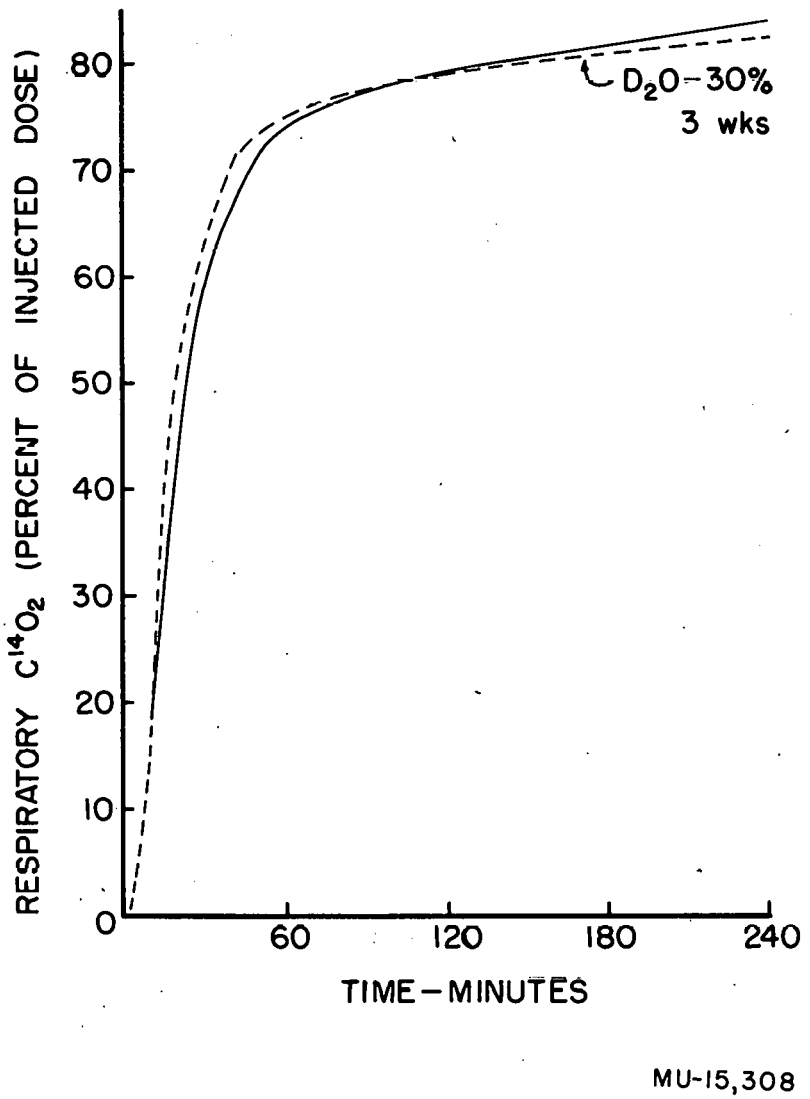


Fig. 13. Cumulative respiratory excretion of $C^{14}O_2$ by C_{57} mice after intraperitoneal injection of formate-C^{14}.

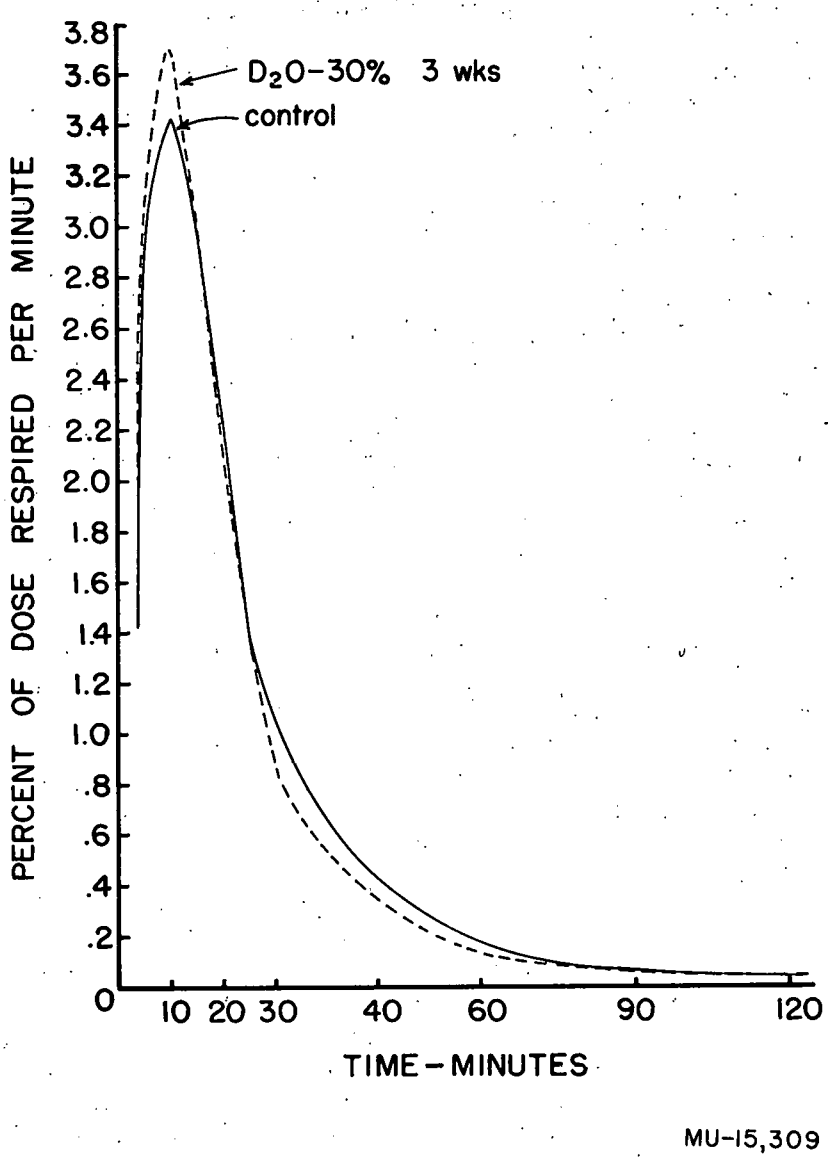


Fig. 14. Rate of respiration of $C^{14}O_2$ by C_{57} mice after intraperitoneal injection of formate-C^{14}.

ELECTRON-SPIN RESONANCE STUDIES OF IRRADIATED CHOLINE CHLORIDE

Robert O. Lindblom and Richard M. Lemmon

Deuteration and electron spin resonance work on irradiated choline chloride has been described in an earlier report.[1] This work indicates that we are dealing with radical ions and electrons rather than pairs of radicals in the chain decomposition of choline chloride. An attempt was made to populate the electron traps and generate radical ions by irradiation of crystalline choline chloride with ultraviolet light. The choline chloride was sealed in quartz ampules under vacuum and irradiated at -196° with a 1-kw mercury-vapor lamp. No electron-spin resonance (ESR) signal was found. Since uv radiation should be energetic enough to excite electrons to the conduction band, failure to observe a signal suggests that the production of radical ions is limited to the number of natural cyrstal defects that can act as traps for conduction electrons, and this number is too small to be observed with ESR. In contrast with uv excitation, 3-Mev electrons can produce crystal defects as well as excite electrons to the conduction band. Fan and Horowitz have shown that energetic electrons can produce crystal defects in germanium, and that 670 kev was the minimum energy necessary to produce defects in germanium.[2]

A dose-saturation effect in the ESR signal from irradiated choline chloride was described in our previous report.[3] Crystalline choline chloride was sealed in Pyrex tubes after evacuation to 100 microns pressure. The samples were kept at -196° during and after irradiation with 3-Mev electrons until the ESR spectrum was determined at room temperature. The dose is calculated from the relationship: dose (in rads) = 1.8×10^5 q (in microcoulombs) which is derived from the Feather relationship

$$\Delta E(\text{Mev}) = \frac{\Delta R}{0.54} \text{ (in g/cm}^2\text{)}.$$

Figures 15 and 16 show the observed relationship of ESR signal to radiation dose. Figure 15 shows a steady increase of ESR signal with dose; however, this plot obscures the decrease in the yield of radicals per unit dose with increasing dose. Figure 16 shows that there is a decrease of 80% in radical yield per unit dose over a 300-fold increase in dose. This drop in yield can best be explained in terms of a radiation-induced annealing of crystal defects.

The kinetics of the radical termination process have been studied by observing the decay of the ESR signal with time (at room temperature) from irradiated choline chloride. If this process were rate-limited by the thermal annealing of traps of one depth, it would show first-order kinetics. A more

---

[1] Robert O. Lindblom and Richard M. Lemmon, in Chemistry Division Quarterly Report, UCRL-8141, Dec. 1957, p. 69.

[2] H. Y. Fan and K. L. Horowitz, reported in Conference on Defects in Crystalline Solids, The Physical Society, p. 232 (1954).

[3] R. O. Lindblom and Richard M. Lemmon, in Chemistry Division Quarterly Report, UCRL-8204, March 1958, p. 5.

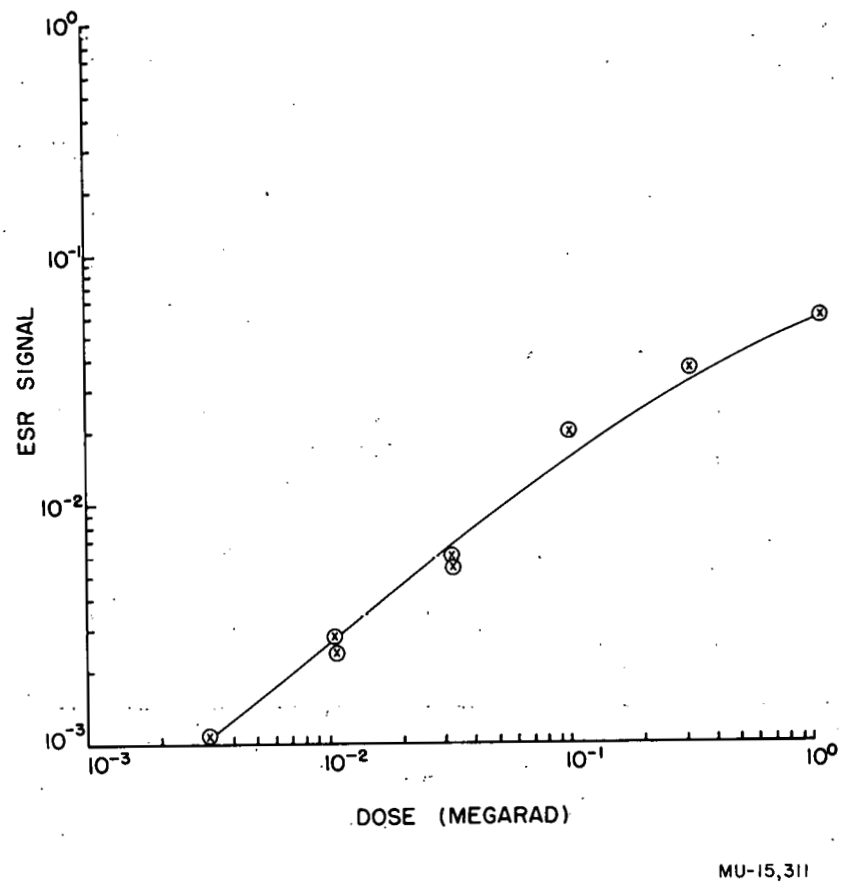


Fig. 15. ESR signal vs dose for irradiated choline chloride.

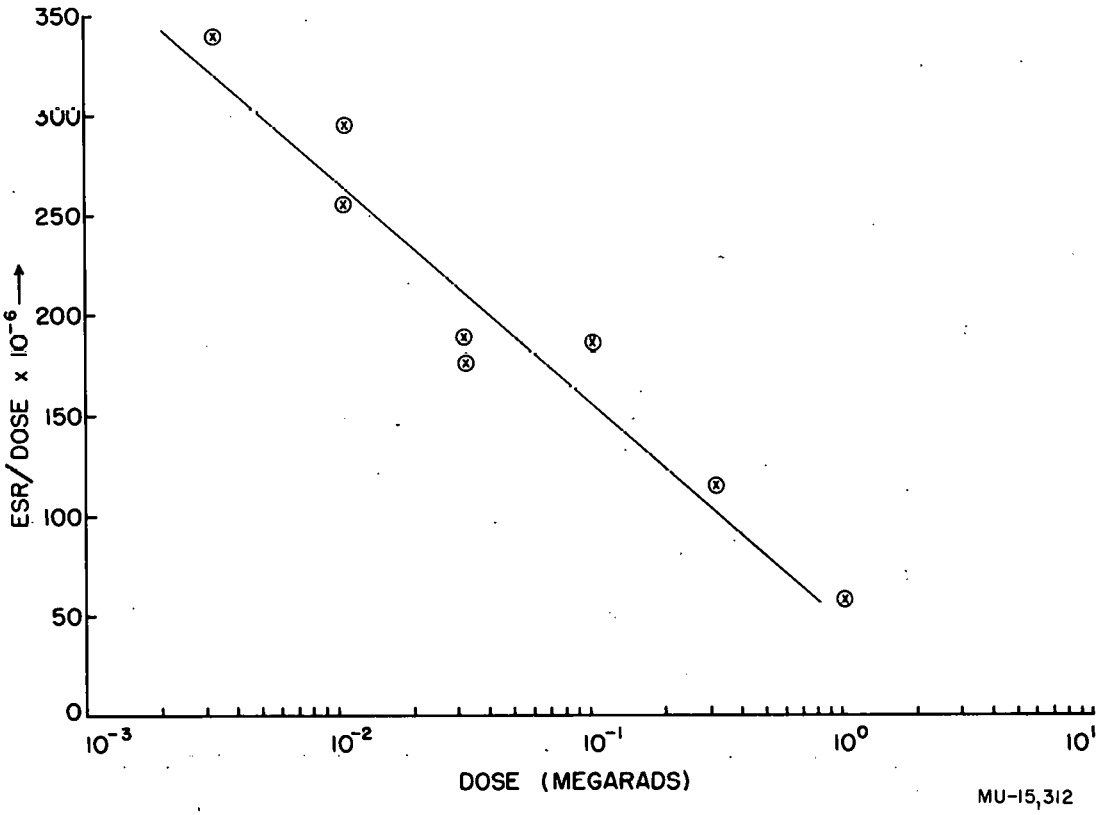


Fig. 16. ESR signal yield vs dose for irradiated choline chloride.

complex decay curve would result if more than one trap depth were involved, or if thermal ionization of trapped electrons without trap destruction were occurring. The decay rate curve shown in Fig. 17 does not fit a first-order plot; moreover, it is suggestive of a two-component curve. Figure 18 shows the same data plotted on log-log paper as the time derivative of the ESR signal versus the ESR signal. On this plot the order of the reaction should appear as the slope of a linear curve for single-rate-constant kinetics. The data best fit a 1-1/2-order rate law, although there is a considerable uncertainty in the data at lower signal levels. Data over a much wider range of values are necessary to resolve the decay kinetics.

THE ISOTOPE EFFECT IN THE DECARBONYLATION OF BENZOYLFORMIC ACID

Bernice Fingerman and Richard M. Lemmon

Two previous reports[1,2] have described studies of the C^{12}/C^{14} isotope effect in the decarbonylation of benzoylformic acid. To supplement this work, the C^{12}/C^{13} isotope effect was studied by using the natural levels of C^{13} present in unlabeled benzoylformic acid synthesized in our laboratory from benzoyl chloride and freshly prepared $Cu_2(CN)_2$. The analyses for C^{13} were performed for us by Dr. Amos Newton of the Nuclear Chemistry Group, using a Consolidated Engineering Corporation Model 12-103 mass spectrometer.

The level of C^{13} in the carboxyl group in our benzoylformic acid was determined on the CO_2 collected from nearly completely (98.5%) decarbonylated benzoylformic acid. On another sample, the CO_2 was collected for the first 10% decarbonylation and also between 90.5 and 93.3% decarbonylation. The level of C^{13} in each of these samples was then determined. The results were as follows: the starting benzoylformic acid contained 1.157 ± 0.003% of C^{13} in the carboxyl position; the first 10% of CO (as CO_2) contained 1.087 ± 0.005% C^{13}; and the fraction 90.5% to 93.3% decarbonylated contained 1.193 ± 0.003% C^{13}.

The C^{12}/C^{14} isotope effect of 10.4% for this reaction was obtained by extrapolation of the data from several determinations back to 0% reaction. The one determination of the C^{12}/C^{13} isotope effect was observed at 10% reaction and showed $(0.070/1.157) \times 100 = 6.1\%$ isotope effect at this point. However, using the formula of Bernstein,[3] and these experimental data, we have calculated a C^{12}/C^{13} isotope effect of 6.3% at 0% reaction. This value is in relatively good agreement with isotope-effect theory,[4,5] which predicts that the C^{12}/C^{14} isotope effect in a given reaction should be about twice the C^{12}/C^{13} value for the same reaction.

---

[1] Bernice Fingerman and Richard M. Lemmon, in Chem. Div. Quarterly Report, UCRL-3950, Sept. 1957, p. 76.

[2] Bernice Fingerman and Richard M. Lemmon, in Chem. Div. Quarterly Report, UCRL-8204, March 1958, p. 9.

[3] R. Bernstein, J. Phys. Chem. 56, 893 (1952).

[4] J. Bigeleisen, J. Chem. Phys. 17, 675 (1949).

[5] J. Bigeleisen and T. L. Allen, J. Chem. Phys. 19, 760 (1951).

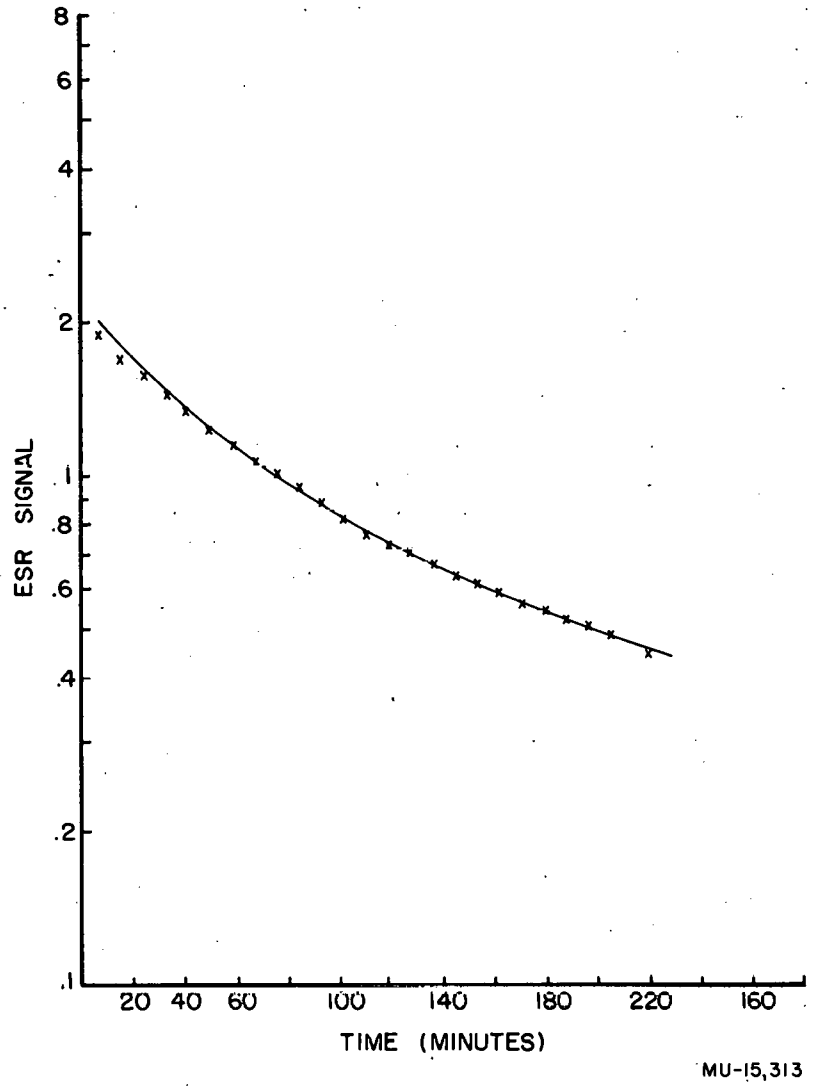


Fig. 17. Time decay of ESR signal from irradiated choline chloride.

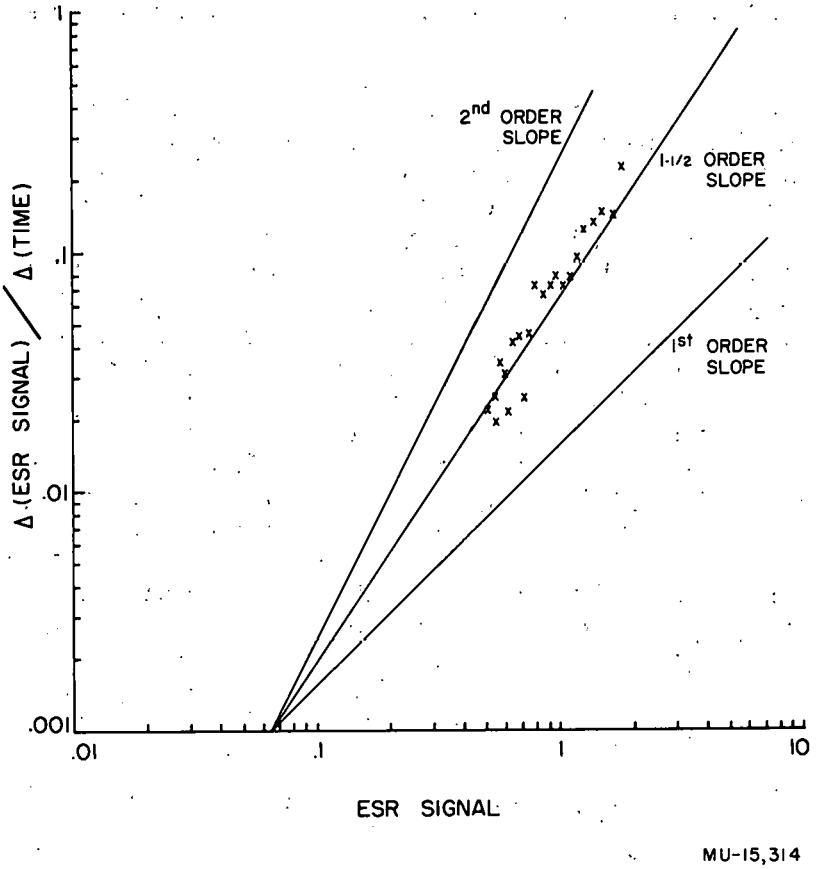


Fig. 18. Reaction order for the decay of irradiated choline chloride.

CARBON-14 ANALYTICAL METHODS

Irville M. Whittemore and Sibyl Kocher

Standardization of the ionization chamber technique for carbon-14 has been completed. It is now possible to take nearly any C^{14}-containing compound, convert it to CO_2, and determine its specific activity with a standard error of less than 1% or 40 disintegrations per minute, whichever is larger.

All but one of the methods used to convert the carbon compounds to CO_2 and measure the activity have been described elsewhere.[1-7] The new method concerns the conversion of alkali salts of organic acids completely to CO_2 in a sealed tube. It has been observed that $CuSO_4$ in a sealed tube at 650°C could be used as an acid oxidizing agent for these salts, and that quite reproducible results could be obtained by this method.[4] However, SO_2 is formed in this reaction and is introduced into the ionization chamber. Wilkenson has pointed out that SO_2 is an electro-negative gas and may lead to a lack of complete collection of the electrons when present in an ionization chamber.[8] Earlier tests indicated that the effect of the SO_2 was negligible. However, recent carefully controlled experiments have shown that when samples of the order of 10 mg are oxidized with $CuSO_4$ and the resultant gas mixture introduced into a 250-ml ionization chamber, the results are about 2% lower than theoretical based on a standard combustion method. In order to avoid this source of error, a different method of oxidizing these salts has been devised. An excess of V_2O_5 is added to the sample before evacuating and sealing the tube. This material acts as an oxidizing acid. With sodium acetate, the results with V_2O_5 are higher than those obtained with $CuSO_4$ by the predicted amount, and with standard benzoic acid-C^{14} the results with V_2O_5 are the same as those obtained by the standard combustion method.

---

[1] K. E. Wilzbach and W. Y. Sykes, Science 120, 494 (1954).

[2] G. L. Brownell and H. S. Lockhart, Nucleonics 10, No. 2, 26 (1952).

[3] B. M. Tolbert, Ionization Chamber Assay of Radioactive Gases, UCRL-3499 March 1956.

[4] Irville M. Whittemore and B. M. Tolbert, in Chemistry Division Quarterly Report, UCRL-3415, June 1956, p. 45.

[5] Irville M. Whittemore, E. Ann Ludwigsen, and Bert M. Tolbert, in Chemistry Division Quarterly Report, UCRL-3595, Oct. 1956, p. 12.

[6] Irville M. Whittemore, Bert M. Tolbert, and E. Ann Ludwigsen, in Chemistry Division Quarterly Report, UCRL-3629, Jan. 1957, p. 29.

[7] I. M. Whittemore, B. M. Tolbert and E. A. Ludwigsen, in Chemistry Division Quarterly Report, UCRL-3836, June 1957, p. 12.

[8] D. H. Wilkenson, Ionization Chambers and Counters (University Press, Cambridge, 1949).

This method of oxidation has worked well with a wide variety of C^{14}-labeled sodium salts. However, when small amounts of activity (a few thousand dpm) were analyzed by this method, the results obtained were high. Blank determinations showed a very high background, apparently due mainly to alpha-particle events. This is not unreasonable, as a large portion of the current vanadium supply is derived from carnotite, a complex mineral containing uranium as one of its components. From our samples this background seems to be constant within a single lot of V_2O_5. If the oxidizer is weighed carefully for all low samples, a correction may be made for the increased background.

We have also reported a method quantitatively releasing CO_2 from $BaCO_3$ in a sealed tube at 650° C, using $CuSO_4$ as the acid.[5] We investigated the products of this reaction, using gas chromatography. No trace of SO_2 was found. It is estimated that the analytical procedure would have been capable of detecting as little as 0.1% SO_2 in the products introduced into a 250-ml ionization chamber. We therefore concluded that this method of CO_2 production from $BaCO_3$ was not subject to the error noted when $CuSO_4$ was used as an oxidizing agent.

ENZYMIC STUDIES OF THE RAT BRAIN

Edward L. Bennett, Hilda Karlsson, Nancy Dye, Ann Ohlander, and Thomas H. Roderick

(In collaboration with Professors David Krech and Mark R. Rosenzweig, Department of Psychology, and Professor E. R. Dempster, Department of Genetics)

During the past four years we have been investigating possible relationships between brain chemistry and behavior. Previous quarterly reports have summarized some of the results we have obtained.[1-6] Our primary objective in these investigations has been the normal enzymic control of behavior, and such studies, correlational in nature, depend upon accurate measurement of normal individual differences in enzymic activity. In the course of these investigations we have accumulated considerable data on individual differences in brain cholinesterase (ChE) activity, lactic dehydrogenase (LDH) activity, and brain weight in the rat as functions of age and strain. These results and some recent results of the genetic breeding program[4-6] are summarized in this report.

A. Individual, Strain and Age Differences in Cholinesterase Activity of the Rat Brain

Previous studies of growth curves of ChE in the brain of the rat have been primarily concerned with changes during the first month after birth, and changes after that time are usually based upon very few determinations. Little information has been published on the variability among the experimental animals. No information has been available to determine possible strain differences and hence the role of genetic factors in brain enzyme activity.

---

[1] Edward L. Bennett and Barbara Krueckel, in Chemistry Division Quarterly Report, UCRL-2709, Sept. 1954, p. 4.

[2] Edward L. Bennett, Barbara Krueckel, and A. Halevy, in Chemistry Division Quarterly Report, UCRL-3157, Sept. 1955, p. 28.

[3] Edward L. Bennett, in Chemistry Division Quarterly Report, UCRL-3351, March 1956, p. 14.

[4] Thomas H. Roderick, Edward L. Bennett, and Hilda Karlsson, in Chemistry Division Quarterly Report, UCRL-3950, Sept. 1957, p. 86.

[5] Hilda Karlsson and Edward L. Bennett, in Chemistry Division Quarterly Report, UCRL-3950, Sept. 1957, p. 93.

[6] Thomas H. Roderick, Edward L. Bennett, Hilda Karlsson, and Ann Ohlander, in Chemistry Division Quarterly Report, UCRL-8141, Dec. 1957, p. 62.

The cortical ChE activity of rats of the S_1 and S_3 strains of rats obtained from the Department of Psychology colony has been determined as a function of age from 29 to 500 days. Data from 432 rats obtained over a 3-year period are summarized in Table VIII. Data from all animals sacrificed within a 10-day interval, starting with an integral multiple of 10, have been grouped together. For each age group the mean age and the mean, standard deviation, and range of ChE values have been computed.

As shown in Fig. 19, cortical ChE activity increases rapidly until about 60 days of age, and the rate of increase falls off until a broad maximum is reached at about 100 days of age. Thereafter ChE activity per unit weight of tissue decreases slowly and continuously, reaching at approximately 500 days the level it had at 40 days. However, the weight of the dorsal cortex slowly increases after 100 days, and the total cortical ChE activity, (cortical ChE activity per unit weight) $\times$ (cortical weight), remains essentially constant after 100 days. At every age, the mean ChE values for the S_1 strain are higher than those for the S_3 strain; the mean difference is about 10%. The difference is statistically highly significant.

Data for the subcortical ChE activity for the two strains of rats are presented in Table IX for 275 rats ranging in age from 28 to 131 days. The maximum subcortical activity is found at about 80 days (Fig. 20), about 20 days earlier than was found for the cortex. The ChE activity of the S_1 strain is approximately 6% greater than for the S_3 strain of rats. Brain weight also increases with age, and, as was found for the cortex, total subcortical ChE remains essentially constant after 80 days.

The coefficients of variation of cortical ChE, subcortical ChE, and brain weight are less than 6. These data indicate that individual differences in brain chemistry are probably smaller than those found in other tissues.

B. Cholinesterase and Lactic Dehydrogenase Activity

The acetylcholine-ChE system is only one of numerous biochemical systems that are important in the metabolism of the brain. Thus the correlations that have been found between ChE activity of the cortex and the behavior of the rat may be nonspecific; that is, ChE activity may simply reflect the general enzyme and metabolic levels in the rat brain. It is therefore of interest to determine what relationships exist between ChE and other enzyme systems. We have studied the lactic dehydrogenase (LDH) activity in the rat brain. If the LDH and ChE systems are quite unrelated, and if no correlation is found to exist between LDH and ChE, this will support the hypothesis that the observed correlation between cortical ChE and adaptive behavior does not simply reflect a correlation between general metabolic rate in the brain and adaptive behavior, but, rather, is specific to the acetylcholine-ChE system. The results of LDH analyses of two areas of the cortex and the subcortical brain of 106 rats of the S_1 and S_3 strains are summarized and compared with determinations of ChE activity in these same rats in Fig. 21. Significant differences between ChE activity and LDH activity in the rat brain are evident and are summarized below.

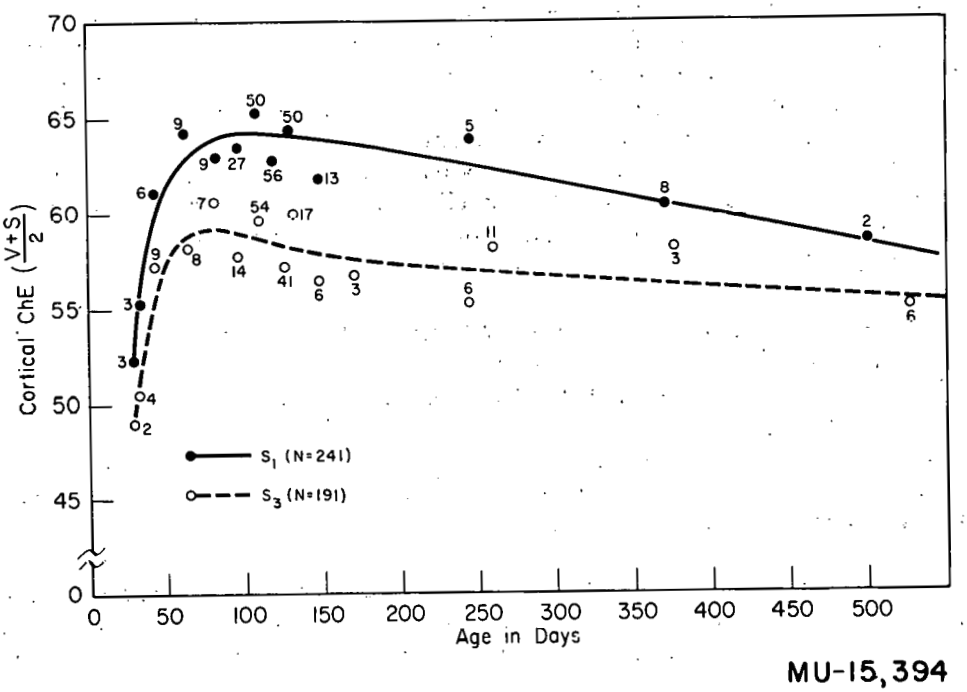


Fig. 19. Cortical cholinesterase (ChE) activity of the S_1 and S_3 strains of rats as a function of age.
The ChE values are based on an average of samples obtained from the visual and somesthetic areas of the cortex. They are expressed in moles ACh $\times 10^{10}$ hydrolyzed per minute per mg of tissue. The number at each point shows the number of animals upon which the value is based.

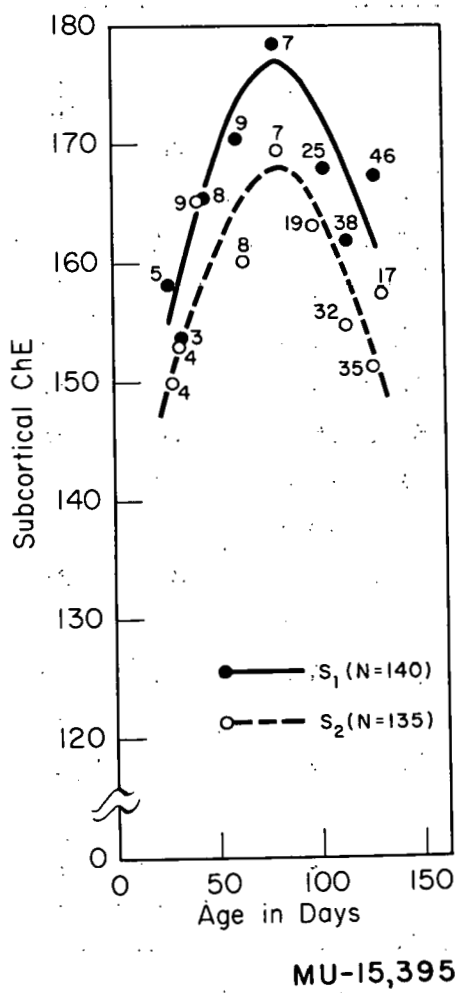


Fig. 20. Cholinesterase (ChE) activity in the subcortical brain of the S_1 and S_3 strains of rats as a function of age. The ChE values are for brains from which the dorsal cortex had been removed. They are expressed in moles ACh $\times 10^{10}$ hydrolyzed per minute per mg of tissue. The number at each point shows the number of animals upon which the value is based.

Table VIII

Mean, standard deviation, and range of cortical cholinesterase activity, as a function of age, for S_1 and S_3 strains of rats.

S_1					S_3				
N	Age in days	Mean ChE $\frac{V+S}{2}$[a]	S.D.	Range	N	Age in days	Mean ChE $\frac{V+S}{2}$[a]	S.D.	Range
3	29	52.2	1.54	50.6-54.3	2	29	49.0	2.40	46.6-51.4
3	34	55.3	1.19	53.8-56.7	4	32	50.5	.83	49.4-51.6
6	43	61.1	2.80	55.8-64.8	9	43	57.2	3.89	54.0-67.6
9	62	64.2	2.84	61.4-69.2	8	64	58.2	3.15	53.8-63.1
9	82	63.0	3.06	58.0-68.4	7	81	60.6	4.60	50.6-64.8
27	96	63.5	4.35	55.0-75.0	14	96	57.7	3.28	53.6-65.0
50	108	65.3	3.95	58.6-72.4	54	110	59.6	3.47	52.7-69.0
56	118	62.8	3.16	56.2-69.7					
50	128	64.4	3.83	52.0-72.8	41	126	57.2	3.45	51.2-63.6
					17	131	60.0	2.92	55.3-64.6
13	147	61.8	3.95	55.0-73.0	6	147	56.5	2.14	52.4-58.4
					3	171	56.7	5.73	50.0-64.0
5	245	63.9	3.06	59.2-68.4	6	244	55.3	2.14	51.3-58.0
					11	260	58.2	5.49	51.0-67.0
8	370	60.4	4.32	55.7-68.0	3	376	58.1	2.73	54.2-60.1
2	500	58.5	2.50	56.0-61.0	6	527	55.0	1.29	53.0-57.0

[a] Cholinesterase activity was averaged for samples of visual and somesthetic regions of cerebral cortex and is expressed in moles ACh $\times 10^{10}$ hydrolyzed per minute per mg of tissue.

Table IX

Mean, standard deviation, and range of subcortical cholinesterase activity, as a function of age, for S_1 and S_3 strains of rats.

S_1					S_3				
N	Age in days	Mean ChE[a]	S.D.	Range	N	Age in days	Mean ChE[a]	S.D.	Range
5	28	158.2	8.33	144-169	4	29	150.0	7.58	142-159
3	34	153.7	3.09	151-158	4	32	153.0	2.12	150-155
8	44	165.4	3.50	161-173	9	43	165.2	8.51	153-184
9	62	170.3	8.41	154-186	8	64	160.1	5.88	150-167
7	80	178.4	8.91	163-199	7	81	169.3	9.05	159-185
25	104	167.9	9.72	154-192	19	98	163.1	9.28	148-182
38	114	161.8	9.16	137-180	32	114	154.8	11.91	134-180
45	128	167.3	7.65	149-182	35	127	151.2	6.76	140-164
					17	131	157.4	8.59	142-171

[a]Cholinesterase activity is expressed in moles ACh $\times 10^{10}$ hydrolyzed per minute per mg of tissue.

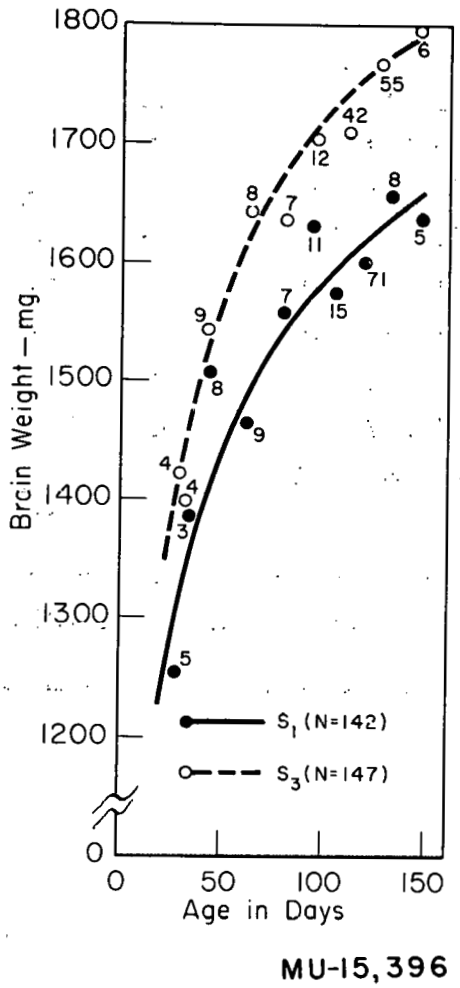


Fig. 21. Boxes A and B give cortical lactic dehydrogenase activity for the S_1 and S_3 strains, respectively. The LDH is expressed in terms of moles $\times 10^9$ DPNH formed/min/mg tissue. The mean LDH activity of the visual area (V) of the cortex is shown by a solid dot, the upper and lower ends of the solid bar indicating ± 1 standard deviation from the mean. The LDH activity of the somesthetic area (S) is similarly shown by an open dot and a dotted bar. For each age, the number of animals is indicated. Since the LDH values for visual and somesthetic areas overlap, the visual values have been displaced slightly to the left and the somesthetic values slightly to the right of the graph.
Boxes C and D give corresponding values for cortical cholinesterase activity expressed in terms of moles $\times 10^{10}$ ACh hydrolyzed/min/mg tissue.

B. Cholinesterase and Lactic Dehydrogenase Activity

The actylcholine-ChE system is only one of numerous biochemical systems that are important in the metabolism of the brain. Thus the correlations that have been found between ChE activity of the cortex and the behavior of the rat may be nonspecific; that is, ChE activity may simply reflect the general enzyme and metabolic levels in the rat brain. It is therefore of interest to determine what relationships exist between ChE and other enzyme systems. We have studied the lactic dehydrogenase (LDH) activity in the rat brain. If the LDH and ChE systems are quite unrelated, and if no correlation is found to exist between LDH and ChE, this will support the hypothesis that the observed correlation between cortical ChE and adaptive behavior does not simply reflect a correlation between general metabolic rate in the brain and adaptive behavior, but, rather, is specific to the acetylcholine-ChE system. The results of LDH analyses of two areas of the cortex and the subcortical brain of 106 rats of the S_1 and S_3 strains are summarized and compared with determinations of ChE activity in these same rats in Fig. 21. Significant differences between ChE activity and LDH activity in the rat brain are evident and are summarized below.

1. The distribution of LDH in the cortex and the subcortex is quite unlike that of ChE in both strains, the ChE activity of the V section is 15% to 20% less than that of the S section, and the average ChE activity of the V and S section is only 35% to 40% of the activity of the subcortical brain. On the other hand, the data presented here indicate that the LDH activity of the V section is the same as or slightly higher than that of the S section, and the average LDH activity of the V and S sections is 110% to 120% of the activity of the subcortical brain. (A similar result was found with a smaller group of animals which were genetically bred for high and low levels of cortical ChE.[5,6])

2. ChE and LDH activity vary differently with age. Cortical ChE activity increases to a maximum at 100 days, whereas LDH activity is essentially constant at all ages tested.

3. As was found for ChE activity, the variability of LDH activity is small. This constitutes further evidence for a low biochemical variability of the brain in comparison with other tissues. Whereas a statistically significant strain difference has been found for the ChE levels of the S_1 and S_3 strains of rats (S_1 rats have higher ChE activity per unit cortex or brain weight), no strain difference has been found for LDH activity. (Also no strain difference in LDH activity has been found in a smaller group of rats bred for high and for low cortical ChE activity.[5]) Since the S_3-strain rats have a larger brain than the S_1-strain rats, the total brain LDH activity of the S_3-strain rat is greater than that of the S_1 strain of rats.

Thus, these results clearly indicate that the distribution of LDH and ChE activities in the cortex and subcortical brain of the rat differ in many respects. A low correlation has been found between the ChE and LDH levels within the individual rat. Therefore, the observed correlation between ChE and adaptive behavior cannot be ascribed to some general metabolic level. To date we have found a relationship between ChE and performance in behavioral tests, but no relationship between LDH and performance.

C. Cholinesterase Levels in Selectively Bred Rats

The selective breeding program has progressed to the sixth generation.[4-6] The ChE activity in the subcortical brain has been determined in numerous animals of this generation. The results, summarized in Table X, indicate that highly significant differences now exist in the ChE levels of the cortex and subcortical brain of these rats. The ratio of activity (Lo strain/Hi strain) is smaller for the cortex (0.72 for the Roderick C strain and 0.81 for the Roderick D strain) than for the subcortical brain (0.81 and 0.91, respectively). A similar relationship has been found for the S_1 and S_3 rats. Lactic dehydrogenase activities have been determined for some rats of the sixth selected generation, and preliminary results indicate that no significant differences exist between the Lo and Hi strains.

From the fifth selected generation obtained from the Dempster foundation stock, additional animals' offspring were obtained and are being used for further behavioral and biochemical experiments. Eleven animals from each line have been analyzed for ChE, and the results are summarized in Table XI. These animals were sacrificed at approximately 110 to 120 days of age and the ChE values are higher than for the animals used to obtain the data in Table X, which were sacrificed at approximately 250 days of age. The differences in ChE levels between the Hi and Lo strains are highly significant in this group of 22 animals, and the ratio of activity in the Lo strain to that in the Hi strain is very similar to the ratio found for the sixth generation of Roderick D Hi and Roderick D Lo rats. Further behavioral and biochemical experiments are planned with these selected strains of rats.

Table X

ChE activity of rats selected for high and for low cortical ChE activity. Sixth selected generation.

Statistical measures	Brain Section				Brain Section			
	V	S	$\frac{V+S}{2}$	Subcortical brain	V	S	$\frac{V+S}{2}$	Subcortical brain
	Strain: Roderick C Lo (N = 4 rats)				Strain: Roderick D Lo (N = 8 rats)			
$\bar{X}$	43.6	49.2	46.6	123.8	47.0	50.2	48.6	125.9
σ	4.3	2.3	3.3	3.3	3.8	3.4	2.8	5.8
	Strain: Roderick C Hi (N = 5 rats)				Strain: Roderick D Hi (N = 4 rats)			
$\bar{X}$	61.6	67.4	64.5	152.2	57.1	62.8	60.0	138.5
σ	3.2	2.1	2.5	6.4	1.3	0.7	0.9	1.0
Ratio Lo/Hi	0.71	0.73	0.72	0.81	0.82	0.80	0.81	0.91
Δ_{Hi-Lo}	18.0	18.2	17.9	28.4	10.1	12.6	11.4	12.6
σ_{Hi-Lo}	1.5	1.1	1.5	2.7	1.4	1.3	1.0	2.1
t	12	16	12	10	7.2	9.7	11	6.0
P	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

Table XI

Comparison of ChE activity of offspring from fifth selected generation of Roderick D rats.[a] (Reported in terms of moles acetylcholine hydrolyzed $\times 10^{10}$/min/mg of tissue).

Statistical measures	Brain Section				Brain Section			
	V	S	$\frac{V+S}{2}$	Subcortical brain	V	S	$\frac{V+S}{2}$	Subcortical brain
	Strain: Roderick D Lo (N = 11 rats)				Strain: Roderick D Hi (N = 11 rats)			
$\bar{X}$	53.41	58.26	55.84	136.82	64.17	71.63	67.88	147.27
σ	3.31	2.20	2.48	3.27	3.10	2.04	2.29	4.09
Ratio Lo/Hi	0.83	0.81	0.82	0.93				
Δ_{Hi-Lo}	10.76	13.37	12.04	10.45				
t	7.31	14.4	11.0	6.15				
P	<0.001	<0.001	<0.001	<0.001				

[a] Definitions:

$\sigma = \sqrt{\Sigma(Xn - \bar{X})^2/(N-1)}$ or $= \sqrt{\Sigma x^2/(N-1)}$ = estimated standard deviation of sample population;

$\sigma_{Hi-Lo} = \sqrt{(N_1 + N_2)(x_1^2 + x_2^2)/[N_1N_2(N_1 - 1) + (N_2 - 1)]}$ = standard error of the difference between means.

$t = \Delta_{Hi-Lo}/\sigma_{Hi-Lo}$

P = level of significance .