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BIO-ORGANIC CHEMISTRY QUARTERLY REPORT
December 1959, January and February 1960

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Contents

Similarity Between Fraction I Protein and Protein of the Intralamellar Structure of Spinach Chloroplast . . . .
Roderic B. Park and Ning G. Pon 3

Biodynamics of Carbon Reduction During Photosynthesis . . .
J. A. Bassham and Martha R. Kirk 8

The Effects of Glycolic and Acetic Acids on the $C^{14}O_2$ Fixation Pattern in Chlorella . . . . . .
Gabriel Gingras 13

Degradation of Glutamic Acid . . . . . .
Teruko Sato and J. A. Bassham 17

The Chemical Effects of Ionizing Radiation on Nucleotides . .
Cyril Ponnamperuma, Richard M. Lemmon, and Edward L. Bennett 27

Experiments on the Degradation of Toluene. . . . . .
R. Terrence Mullen and Richard M. Lemmon 29

Examination of a Liquid Scintillation Counting Method for Carbon Dioxide-C^{14} . . . . . .
Fritz H. Woeller and Richard M. Lemmon 35

Effects of Environmental Complexity and Training on Brain Chemistry . . . . . .
Edward L. Bennett, Hiromi Morimoto, Marie Hebert, and Ann Ohlander 39

Effects of D_2O on Drosophila . . . . . .
Ann M. Hughes 49

Combined Effects of D_2O and Irradiation in the Production of Recessive Lethal Mutations in Drosophila . . . . . .
Ann M. Hughes and Philip E. Hildreth 50

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M. Calvin, Director

Edited by J. A. Bassham

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March 18, 1960

SIMILARITY BETWEEN FRACTION I PROTEIN AND PROTEIN OF THE INTRALAMELLAR STRUCTURE OF SPINACH CHLOROPLASTS

Roderic B. Park and Ning G. Pon

In 1952, Wildman showed that up to 50% of soluble green leaf protein has a sedimentation coefficient ($S_{w,20}$) of 16 to 19.[1] This protein fraction was designated as Fraction I. Subsequently Fraction I was shown to have a molecular weight of about 400,000,[2] and to possess carboxydismutase activity.[3] A large proportion of the Fraction I in spinach leaves was found to be associated with the isolated spinach chloroplasts and could be readily released from the chloroplasts by osmotic rupture of the chloroplast membrane.[4]

While studying the relation of structure and size of chloroplast fragments to both Hill-reaction activity and CO_2 fixation activity,[5] we noted, under electron microscopic examination of the shadowed fragments, the presence of characteristic particles 100 A in diameter. These particles appeared to be associated with both the stroma and the lamellar structure of the chloroplast (Fig. 1). The 100-A particles are shown in the background (lower left-hand corner) of the electron micrograph of the large chloroplast fragments. Similar studies of very small chloroplast fragments showed an even larger proportion of these particles. Furthermore, ultracentrifuge studies on the small fragments showed that after the green material had precipitated, two colorless components with sedimentation coefficients about 19 and 46 were present in the supernatant. The similarity between the sedimentation coefficient of Fraction I and that of the $S_{w,20}$-18 component

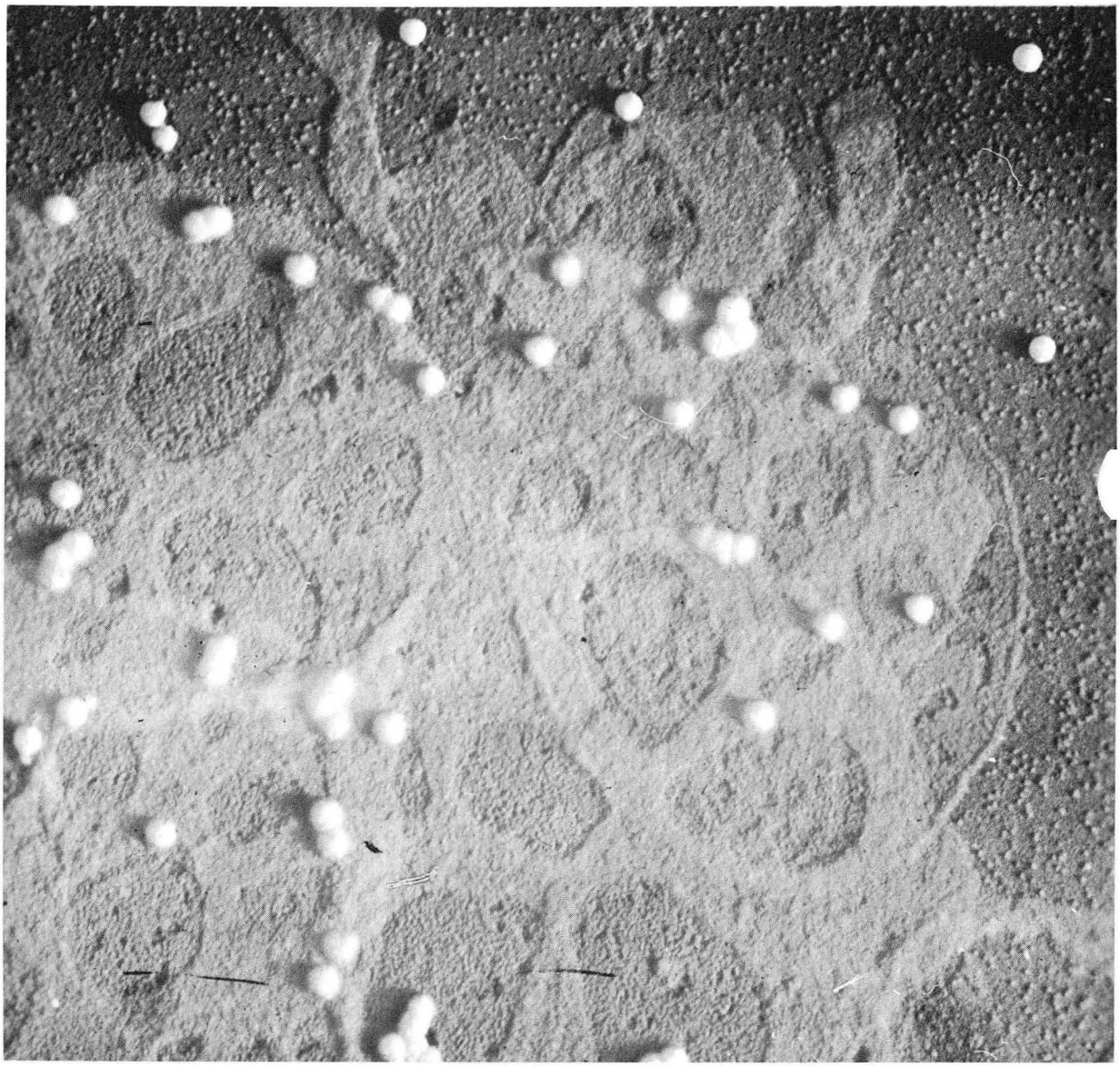
[1] S. J. Singer, L. Eggman, J. M. Campbell, and S. G. Wildman, J. Biol. Chem. 197, 233 (1952).

[2] L. Eggman, S. J. Singer, and S. G. Wildman, J. Biol. Chem. 205, 969 (1953).

[3] J. Mayaudon, Enzymologia 18, 22 (1957).

[4] J. W. Lyttleton and P. O. P. Ts'O, Arch. Biochem. Biophys. 73, 120 (1958).

[5] R. B. Park, K. P. Louwrier, and N. G. Pon, in Bio-Organic Chemistry Quarterly Report, UCRL-8961, Sept. 1959, p. 24.



ZN-2445

Fig. 1. Electron micrograph of spinach chloroplast fragments precipitating between 5,000 and 10,000 × g. (Shadowed with platinum-palladium-gold alloy.) Each large disc, about 20,000 A in diameter, corresponds to a lamellar structure from the chloroplast. The large white spheres are 880-A polystyrene latex.

suggested that the spheres seen under the electron microscope might correspond to Fraction I. To test this hypothesis, Fraction I was prepared from spinach chloroplasts according to the method of Lyttleton and Ts'O.[4] This preparation, having $S_{w,20}$ of 16, was indistinguishable from the 100-A particles shown in Fig. 1 when observed under the electron microscope (Fig. 2).

Our experiments presently indicate that material similar in size to Fraction I is located in two areas of the chloroplast. It is found in the stroma, which is defined here as the colorless medium in which the lamellar structure of the chloroplast is located. Particles the size of Fraction I are also found between layers c as shown in Fig. 3. Spectrophotometric and ultracentrifugal studies show that neither the stroma or lamellar particle contains chlorophyll or carotene pigments. Rather the data indicate that the photosynthetic pigments are contained in layers c of the structure shown in Fig. 3. Many enzymatic activities of the reductive carbon cycle--including carboxydismutase--are, however, associated with the 100-A particles found in the stroma. Whether the sandwiched 100-A particles of layer c are purely for structural purposes or whether they contain certain enzymatic activities is presently under investigation.

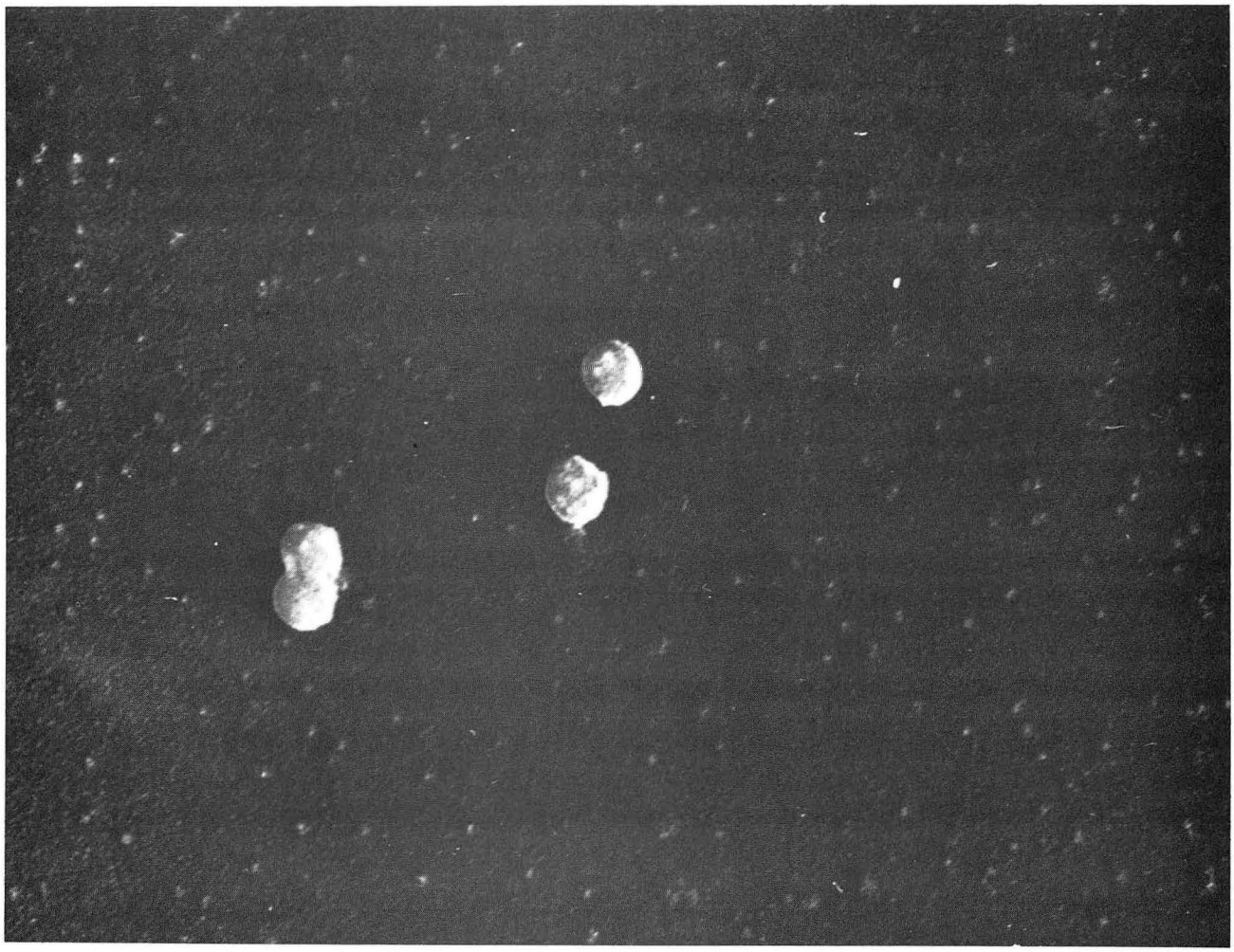


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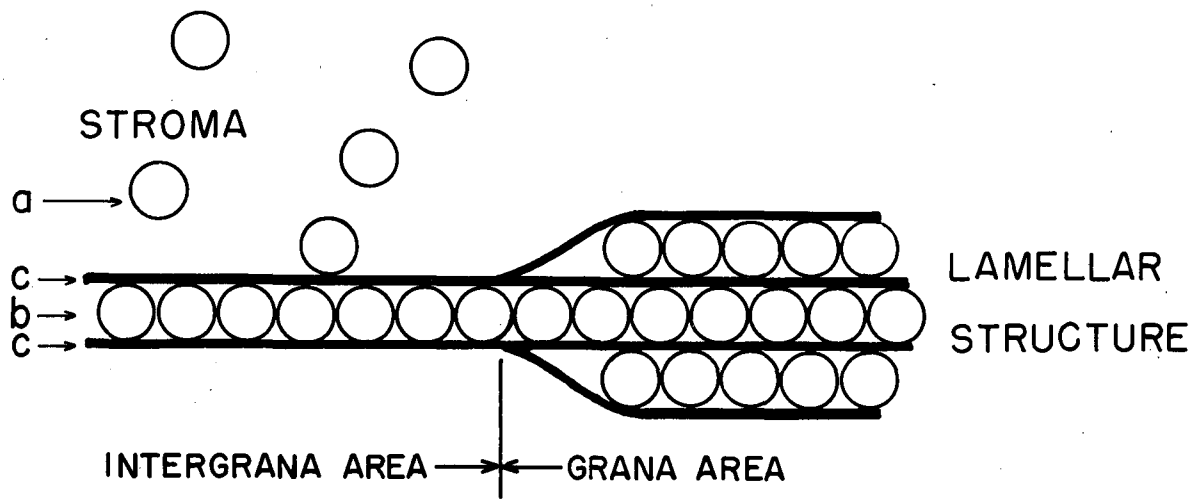
Fig. 2. Electron micrograph of Fraction I protein from spinach chloroplasts shadowed with platinum-palladium-gold alloy. Large white spheres are 880-A polystyrene latex.



MU-19374

Fig. 3. Model of the internal structure of spinach chloroplast.
a. Protein particles (100 A in diameter), which can be released from the chloroplast at low osmotic concentrations, probably Fraction I. The reductive carbon cycle enzymes are associated with these particles.
b. Protein particles (100 A diameter) sandwiched between the pigment-containing layers c.
c. Pigment-containing layer of the lamellar structure.

BIODYNAMICS OF CARBON REDUCTION DURING PHOTOSYNTHESIS

J. A. Bassham and Martha R. Kirk

In previous reports[1, 2, 3] we have described studies of carbon compound transformations during steady-state photosynthesis. In these studies, we have attempted to maintain for several hours a condition of steady-state photosynthesis and growth in Chlorella pyrenoidosa. At the same time we introduced C^{14} as $C^{14}O_2$ and $HC^{14}O_3^-$ into the algal suspension, and (by methods previously described) measured the rate of appearance of C^{14} in various compounds formed by the algae. These measurements permit calculation of a number of biodynamic properties of the system. These include concentrations of metabolic intermediates, and rates of flow of carbon through these intermediates as compared with externally measured rates of CO_2 uptake and oxygen evolution.

These studies have been continued and the conclusions drawn previously have been further confirmed. We are now attempting to obtain more quantitative determinations of the concentrations and rates of synthesis of amino acids. The concentrations of these metabolites are in some cases 10 to 20 times the concentrations of the "primary" intermediates (e.g., phosphoglyceric acid, ribulose 1, 5-diphosphate, etc.), while the rates of flow of carbon through these secondary intermediates are smaller. Therefore, it is necessary to continue the steady-state studies for hours rather than minutes. As mentioned in earlier reports, one must employ the lowest possible concentrations of inorganic salts in the algal medium used during the experiment in order that the subsequent analysis by paper chromatography can be performed. The nutrient solution used in the earlier experiments, while adequate for the purposes of those experiments, was not sufficient to maintain the levels of amino acids and their rates of formation constant over several hours.

We have therefore experimented with various nutrient solutions, testing for the maintenance of steady-state photosynthesis as well as for the effect on paper chromatography. We have now found two solutions which appear to satisfy our requirements. One of these is a "starting" medium (Table I) which is used for suspension of the algae at the start of the experiment. The other is the "adding medium" (Table II) which is added automatically to the algal vessel as the pH of the suspension changes in response to mineral uptake by the algae.[3]

---

[1] J. A. Bassham, M. Kirk, and T. Andrews, in Bio-Organic Chemistry Quarterly Report, UCRL-8698, March 1959, p. 19.

[2] J. A. Bassham and M. Kirk in Bio-Organic Chemistry Quarterly Report, UCRL-8421, June 1958, p. 18.

[3] J. A. Bassham and M. R. Kirk in Bio-Organic Chemistry Quarterly Report, UCRL-8848, June 1959, p. 32.

Table I

Starting medium	
KH_2PO_4	0.20 mM
$MgSO_4 \cdot 7H_2O$	0.20 mM
$MgCl_2$	0.20 mM
K_2CO_3	0.10 mM
$Ca(NO_3)_2$	0.02 mM
KNO_3	0.05 mM
Arnon's A-4[12] solution (Ref. 4) of trace elements plus	
$CoCl_2 \cdot 6H_2O$ (40 mg/l) and MoO_3 (15 mg/l)	1 ml/liter
NH_4VO_3(0.023 g/l)	1 ml/liter
$(NH_4)_2CO_3$	0.15 mM
Saturate with 2% CO_2 by bubbling mixture of	
2% CO_2 in air through solution until pH reaches	
a constant level. Add	
$FeCl_3$	0.02 mM

[4] R. W. Krauss, in Algal Culture from Laboratory to Pilot Plant, ed. by J. S. Burlew, Carnegie Institute of Washington, Publication 600, Washington, D. C., 1953, p. 94.

Table II

Adding medium	
$(NH_4)_2CO_3$	6.75 m$\underline{M}$
$NH_4H_2PO_4$	1.10 m$\underline{M}$
KH_2PO_4	0.40 m$\underline{M}$
$MgSO_4 \cdot 7H_2O$	1.5 m$\underline{M}$
$MgCl_2$	1.5 m$\underline{M}$
KNO_3	0.05 m$\underline{M}$
$Ca(NO_3)_2$	0.05 m$\underline{M}$
Arnon's A-4[12] (Ref. 4) solution of trace elements plus $CoCl_2 \cdot 6H_2O$ (40 mg/l) and MoO_3(15 mg/l)	1 ml/liter
NH_4VO_3 (0.023 g/l)	1 ml/liter
$FeCl_3$	0.1 m$\underline{M}$

In order to assure solution of all the elements in the adding medium it is necessary to dissolve the ammonium carbonate in about 80% of the water and bubble a mixture of 2% CO_2 in air through it until a constant pH is obtained. All the other elements, except the $FeCl_3$, are dissolved in the remaining 20% of water. These two parts are then mixed and the proper amount of a concentrated solution of $FeCl_3$ is added. Even so, when the $FeCl_3$ solution is added to the rest of the adding medium, some cloudiness occurs. However, this precipitate does not settle out during the course of the experiment, and it is presumed that the chemicals contained in the precipitate are available to the algae once it is added to the more acidic algal solution.

When the algae are first suspended in the starting medium the pH is above 6. After 2% CO_2 in air has been passing through the algal suspension for several minutes, the pH drops to about 5.8. The lights are then turned on to illuminate the algae cell and photosynthesis begins. For a few minutes the pH rises, for some unknown reason. When the pH begins to fall owing to NH_4^+ uptake, the pH control unit is set to control at pH = 6 and is turned on. For about 30 minutes the adding medium comes into the cell sporadically but thereafter the rate of addition becomes fairly constant.

Table III shows the rate of uptake of ammonia and CO_2 along with the rate of oxygen evolution when the above media are used over a period of time to maintain the continuous growth of a 1% suspension of Chlorella.

The rate of NH_4^+ uptake is calculated from the rate of addition of the adding medium and the known concentration of NH_4^+ in the adding medium.

Some NH_4^+ is undoubtedly withdrawn when algal suspension is removed from the algal cell to keep the volume of the suspension constant. Therefore, this calculation gives the upper limit of NH_4^+ uptake. Assuming the values in Table III to be nearly correct, the synthesis of protein in this experiment would account for about one-half of all the carbon taken up by the algae.

Table III

Rate of NH_4^+ and CO_2 uptake and O_2 evolution of *Chlorella* grown in described media

Time (hours after lights on)	Rate (μM/min/g wet-packed algae)		
	O_2	CO_2	NH_4^+ [a]
0.5	20.4	22.2	
1.0			2.40
2.0	19.8	21.1	3.26
2.75	20.8	19.3	

[a] upper limit

THE EFFECTS OF GLYCOLIC AND ACETIC ACIDS ON THE $C^{14}O_2$ FIXATION PATTERN IN CHLORELLA

Gabriel Gingras

In previous work at this laboratory it has been observed that glycolic acid is sometimes an important product of CO_2 fixation during photosynthesis.[1] However, it has not been possible to identify its role in photosynthetic carbon metabolism. In an attempt to learn more about this probelm, glycolic acid has been administered to Chlorella photosynthesizing in the presence of $C^{14}O_2$. It was reasoned that the unlabeled glycolic acid would dilute the radioactivity of its first metabolites, thereby suggesting their chemical nature. To ascertain the specificity of the effects thus obtained a companion experiment was run in which acetate was the source of C^{12}.

Experimental Procedure

Chlorella cells were incubated for one hour in dim light at room temperature, in a solution (adjusted to pH 6.3) containing 5×10^{-4} M ammonium phosphate and 1×10^{-2} M glycolic or acetic acid. One percent CO_2 in air was bubbled through the 1% algal suspension. After the incubation period, 400 μC of $NaHC^{14}O_3$ (26 μM) was added to 10.0 ml of suspension. Photosynthesis was carried out in flattened tubes 6 in. from a General Electric RSP 2 photospot. A water-cooled infrared filter was used to prevent excessing heating. Different periods of light or dark or both were given as indicated in Tables IV and V. The algae were killed and analyzed as indicated in a previous work.[2]

Results

Table IV shows the results obtained with glycolic acid and Table V the results obtained with acetic acid in the incubation medium.

Table IV shows that glycolic acid reduces the radioactivity in the lipids and that it affects the unsaponifiable fraction rather than the fatty acids. As would be expected, the radioactivity in glycolic acid itself and in serine is also decreased. Aspartic, malic, and fumaric acids show also a low radioactivity in the presence of unlabeled glycolic acid, under certain conditions.

Table V shows that acetic acid, in contrast to glycolic acid, lowers the radioactivity in the fatty acids moiety to a considerably greater extent than in the unsaponifiable fraction of the lipids. Unlabeled acetic acid also lowers the radioactivity in malic, fumaric, and aspartic acids, whereas it increases the radioactivity in glycolic, citric, glutamic, and (perhaps) succinic acids. This effect can be explained if the rate of condensation of oxaloacetic acid with acetate is increased in the presence of added acetate.

---

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

[2] G. Gingras and J. A. Bassham, in Bio-Organic Chemistry Quarterly Report, UCRL-8457, Sept. 1958, p. 32-38.

Table IV

Effect of unlabeled glycolate on the $C^{14}O_2$ fixation pattern

	1L[a]	1L+G	1L, 5D	1L, 5D+G	6D	6D+G	6L	6L+G
Total fixation (cpm $\times 10^{-6}$)	11.8	10.1	11.0	9.3	1.2	0.9	61.4	56.8
Lipids (cpm $\times 10^{-3}$)	512	306	1068	632	121	28	4290	4800
Fatty acids (cpm $\times 10^{-3}$)	116	103	169	167	3.8	4.2	1742	2380
Unsaponifiable acids (cpm $\times 10^{-3}$)	396	203	899	465	117	24	2548	2412
PGA[b]	25.4	25.8	7.0	5.9	3.8	4.8	6.3	5.9
PEPA	0.3	0.3	0.1	0.1	0.2	0.3	0.3	0.1
Alanine	12.3	9.8	16.3	16.4	3.6	4.3	21.7	23.9
Malic acid	8.8	5.7	1.5	1.2	3.6	4.9	15.9	15.8
Citric acid	0.1	0.2	0.3	0.3	0.4	0.6	1.1	1.1
Fumaric acid	1.9	0.9	0.4	0.4	0.9	1.2	4.3	3.8
Glycolic acid	~ 0	~ 0	0.6	~ 0	~ 0	~ 0	0.7	0.1
Aspartic acid	7.8	5.2	23.0	25.5	66.9	63.2	8.4	6.1
Glutamic acid	0.2	0.3	17.2	16.1	14.9	15.1	5.4	6.1
Serine	2.3	1.5	5.3	2.5	-	-	5.0	4.2
Glycine	0.3	0.4	0.6	0.3	-	-	1.1	1.6

[a]The following abbreviations are used: L = light
D = dark
G = glycolate

Example, 1L, 5D+G = 1 minute light followed by 5 minutes dark in the presence of glycolate.

[b]Figures hereafter in percent of radioactivity fixed in the soluble fraction.

Table V

Effect of unlabeled acetate on the $C^{14}O_2$ fixation pattern

	1L[a]	1L+Ac	1L, 5D	1L, 5D+Ac	6D	6D+Ac
Total fixation (cpm $\times 10^{-6}$)	23.1	14.1	15.6	12.6	1.3	0.8
Soluble fraction (cpm $\times 10^{-6}$)	19.5	11.5	15.5	12.1	1.3	0.5
Lipids (cpm $\times 10^{-3}$)	1552	486	1630	804	78	13
Fatty acids (cpm $\times 10^{-3}$)	468	83	634	117	4.6	2.8
Unsaponifiable acids (cpm $\times 10^{-3}$)	108	40	996	687	73	10
PGA[b]	19.6	21.1	6.0	7.7	5.6	4.2
PEPA	0.3	0.5	0.3	0.4	0.4	0.4
Alanine	8.1	5.9	9.2	9.7	4.5	3.8
Malic acid	15.5	11.1	4.2	4.3	7.1	6.0
Fumaric acid	2.9	2.2	1.0	1.1	1.7	0.9
Glycolic acid	1.4	3.0	~ 0	1.5	~ 0	~ 0
Aspartic acid	7.2	6.4	26.5	12.7	50.4	16.9
Glutamic acid	1.5	1.6	18.2	22.9	17.2	44.0
Citric acid	0.5	1.6	0.7	4.8	1.1	6.5
Succinic acid[c]	~ 0	~ 0	~ 0	1.5	~ 0	1.3

[a] The following abbreviations are used: L = light
D = dark
Ac = acetate

Example, 1L, 5D+Ac = 1 minute light followed by 5 minutes dark in the presence of acetate

[b] Figures hereafter in percent of radioactivity fixed in the soluble fraction.

[c] Tentative identification.

This would cause a decrease in the concentrations of malic, fumaric, and aspartic acids accompanied by an increase in concentrations of citric, glutamic, and succinic acids. The increased radioactivity in glycolic acid could be ascribed to the breakdown of a greater quantity of isocitrate to give glyoxylate and succinate.

Both glycolate and acetate reduce the total fixation of C^{14} (see especially 1L+Ac). This may be due to (a) a dilution of the radiocarbon by the exogenous source of C^{12} and (b) a slight toxicity of glycolate and acetate at this concentration (10^{-2} M), or to a combination of (a) and (b).

DEGRADATION OF GLUTAMIC ACID

Teruko Sato and J. A. Bassham

Introduction

When one feeds $C^{14}O_2$ to photosynthesizing algae in the presence of NH_4^+ a large amount of labeled glutamic acid is obtained.[1] The way in which the glutamic acid is formed is not known. One approach to learning how glutamic acid is made is to determine the distribution of the label of each carbon atom in glutamic acid as obtained from algae after photosynthesis with $C^{14}O_2$. Such a determination might suggest the metabolic relations of other photosynthetic intermediates to glutamic acid. For example, if glutamic acid is formed from γ-aminobutyric acid, C_1 of glutamic acid will be most labeled after short periods of photosynthesis with $C^{14}O_2$. Another route to glutamic acid that has been suggested is via citric acid and α-ketoglutaric acid. In this case, the citric acid could come from condensation of acetyl CoA with oxaloacetic acid, each derived from photosynthetically produced PGA (Fig. 4). Other as yet unknown pathways, such as $C_2 + C_3$ condensations, may be more important.

From the degradation studies, we should be able to make some choice between these routes.

H. Simon[2] reported previously on the study of degradation of glutamic acid in this laboratory. For the degradation of glutamic acid, he employed the procedure shown in Fig. 5. In his work the radioactivity measurements for the determination of radioactivity were done by measuring CO_2 in ionization chambers. The organic material was combusted in sealed tubes with CuO according to the method of Wilzbach.[3] Carbon dioxide from degradations was trapped in sodium hydroxide solution and converted to $BaCO_3$. The CO_2 from $BaCO_3$ was liberated by $CuSO_4$ at 650° and transferred into an ionization chamber. The Schmidt degradation of glutamic acid-5-C^{14} and glutamic acid-1-C^{14} were checked by Simon. For glutamic acid-5-C^{14}, the yield of CO_2 was about 60 to 70%. The $BaCO_3$ had only 95.1% of the activity in the glutamic acid. In the Schmidt reaction of glutamic acid-1-C^{14}, 6% of the activity was found in $BaCO_3$. In the second step, i.e., the oxidative decarboxylation of α, γ-diaminobutyric acid-1-C^{14} to β-alanine, a yield of about 40% of CO_2 was obtained. The specific activity of CO_2 from this reaction was only 58.4% of the theoretical. The yield of the reaction from β-alanine to acetic acid was also poor, about 5 to 10%. For these reasons, we have sought a better degradation procedure.

---

[1] O. Holm-Hansen, K. Nishida, V. Moses, and M. Calvin, J. Exptl. Botany 10, 109 (1959).

[2] H. Simon, in Chemistry Division Quarterly Report, UCRL-3950, Sept. 1957, p. 13.

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

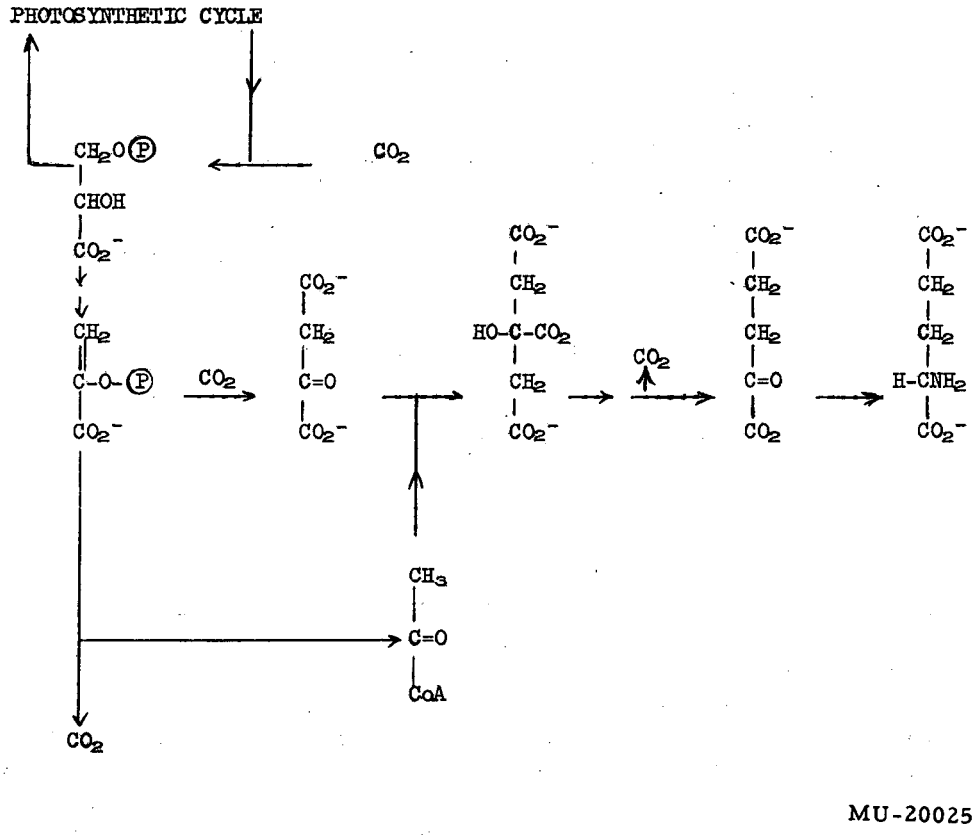
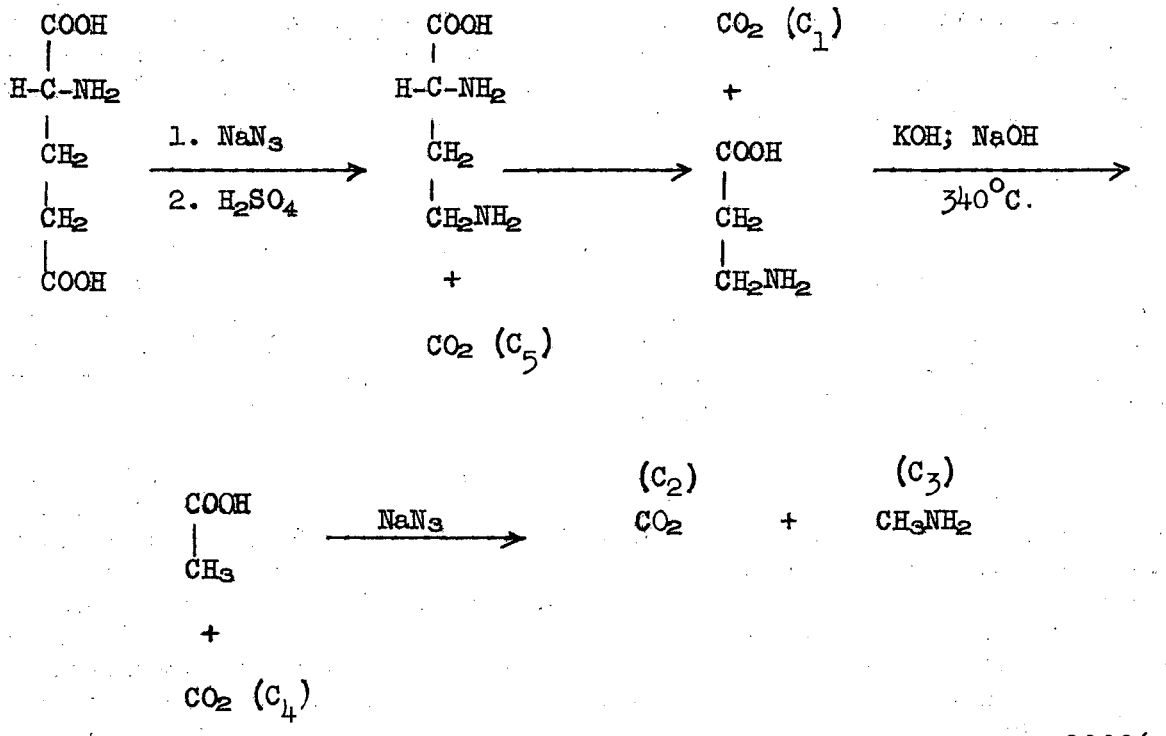


Fig. 4. Photosynthetic carbon fixation pathways.



MU-20026

Fig. 5. Degradation of glutamic acid — Simon.

Experimental Procedures

Source of Radioactive Glutamic Acid

Labeled glutamic acid used for the preliminary test was DL-glutamic acid-1-C^{14} (specific activity 1.32 mC/mM) and DL-glutamic acid-2-C^{14} (specific activity 0.47 mC/mM) obtained commercially. The uniformly labeled glutamic acid used for the degradation study was obtained by paper chromatography of algae extracts after the Chlorella had been allowed to photosynthesize with $C^{14}O_2$. For this photosynthesis a 1/2% suspension of Chlorella in a high ammonia medium was allowed to grow for 80 minutes at approximately light saturation[4] in a specific activity of 4.5 mC/mM.

Degradation Method

The degradation of glutamic acid was attempted according to the method of Mosbach, Phares, and Carson.[5,6] The steps in the degradation procedures are as shown in Fig. 6. In Step 1 glutamic acid is converted to succinic semialdehyde by the method of Dakin,[7] that is, glutamic acid is decarboxylated by using 50% excess of hypochlorous acid, liberated from chloramine T, as the oxidizing agent.

The succinic semialdehyde formed in this reaction is reduced to butyric acid by the Wolff-Kishner reaction. Butyric acid is decarboxylated by the Schmidt reaction. The acid in the presence of 50% excess sodium azide is converted to the azide which then undergoes a rearrangement to give the carboxyl group as carbon dioxide and the balance of the molecule as the amine with one less carbon. Butyric acid was recovered from the reaction mixture by steam distillation, and purified by gas chromatography on a Yucon (polyethylene oxide) column. Propylamine was removed after the Schmidt reaction by distillation from strong alkaline solution, and collected in 0.2 N H_2SO_4 solution. The propylamine sulfate solution was oxidized by 5% $KMnO_4$ aqueous solution.

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[4] J. A. Bassham and Martha R. Kirk, this report, page 7.

[5] E. F. Phares, Arch. Biochem. Biophys. 33, 173 (1951).

[6] E. H. Mosbach, E. F. Phares and S. C. Carson, Arch. Biochem. Biophys. 33, 179 (1951).

[7] H. D. Dakin, Biochem. J. 11, No. 2, 79 (1917).

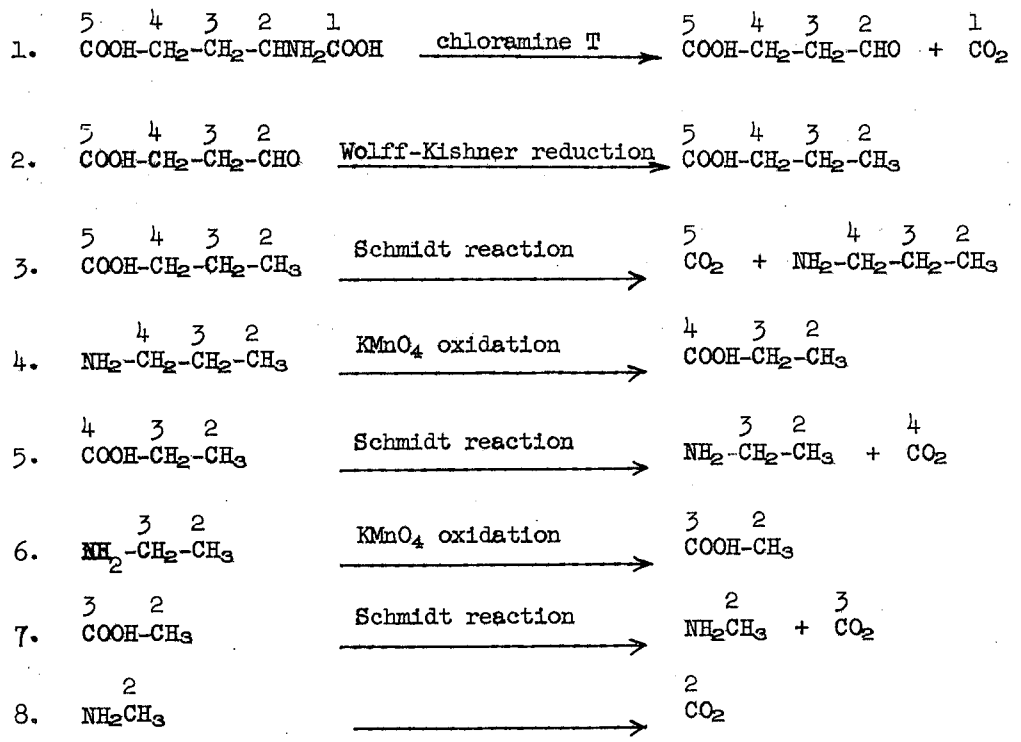

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Fig. 6. Degradation of glutamic acid – Mosbach, Phares, and Carson.

Counting Technique

The counter used was the liquid scintillation counter "Tri-Carb," operated in the prescribed manner. For aqueous samples scintillation solution No. 2 was used: toluene, 2.0 liters; p-dioxane, 2.0 liters; abs. EtOH, 1.2 liters; naphthalene 260 g; PPO 26 g; POPOP 0.5 g. For suspended $BaCO_3$ the solution consisted of thixcin dissolved in scintillation solution No. 6: For suspended $BaCO_3$ toluene, 3.0 liters; PPO, 13.5 g; POPOP, 0.3 g. Thixcin was added in the ratio 100 g to 2.5 g of scintillation solution No. 6. The aliquots of radioactive material to be counted were pipetted accurately with a micropipet into glass vials, which contained 10 ml of scintillation solution No. 2 or No. 6, and counted in the machine. The first count, minus the background of a blank sample, is termed C_1. The variability of the composition of the sample can be controlled by the use of an internal standard. The C^{14} internal standard used was toluene-1-C^{14}, which had been distilled and diluted to an appropriate concentration. One hundred μl of toluene-C^{14} (1.4×10^5 dpm/ml) was pipetted into the counted sample and the vial recounted; this count minus the background is termed C_2.

$$\text{Efficiency of counting} = \frac{C_2 - C_1}{\text{Std. activity added}} = \frac{C_2 - C_1}{1.4 \times 10^4}$$

$$\text{d/m of sample} = \frac{C_1}{\text{efficiency}}$$

Experimental Detail and Results

1. Chloramine T oxidation step

First the chloramine T oxidation of glutamic acid was repeated under various conditions to get the best yield. The following conditions were found to give 88 to 92% yield. In every experiment, 1 g of glutamic acid was used.

One gram (1/147 mole) of glutamic acid and 50 to 60 ml of H_2O were placed in a 250-ml three-necked flask. The pH of this solution was adjusted to 6.5 to 7 with carbonate-free 2 N NaOH, and the glutamic acid was dissolved completely by stirring. Then 2.8785 g (1/147 × 1.5 mole) of chloramine T in 25 ml of H_2O was added at 25 to 30° in a period of 20 min, with stirring and with N_2 gas passing through the system. After all the chloramine T was added, the bath temperature was gradually raised to 50°C over a 30-min period, and this temperature was maintained for 15 min. Then the reaction mixture was cooled to room temperature and 2 N H_2SO_4 added to bring the solution below pH 4. Nitrogen gas flow was maintained during all this period and continued for 2 hours after acidification. The outflowing gas was bubbled through carbonate-free 2 N NaOH to collect the CO_2 evolved. The trapped CO_2 in the NaOH solution was precipitated by adding saturated $BaCl_2$ solution, giving a yield of 1.2319 g of $BaCO_3$, equal to 91.8% of

theoretical. The reaction mixture was cooled in an ice bath, and the precipitate of p-toluene sulfonamide was filtered off. Succinic acid semialdehyde was identified as the 2, 4-dinitrophenylhydrazone. dp 198°. Yield: 1.537 g, 80.3% of theoretical.

In order to determine the blank of $BaCO_3$ obtained in this procedure, the following experiments were carried out.

a. For the purpose of checking $BaCO_3$ from air, an experiment was carried out with the same procedure under almost the same conditions, but without glutamic acid. The pH of H_2O used was 6.1 (without glutamic acid) and 14.4 mg of $BaCO_3$ was obtained. This is 1.07% of the theoretical amount of $BaCO_3$ that 1 g of glutamic acid should give.

b. Instead of glutamic acid, 57.1 mg of $NaHCO_3$ was used. This amount of $NaHCO_3$ gives the same amount of $BaCO_3$ as does 1 g of glutamic acid. The pH of the $NaHCO_3$ solution was 8.33. The yield of $BaCO_3$ was 1.3521 g (100.7% of theoretical yield) or, considering Exp. a, 100.7-1.07 = 99.6%.

c. This experiment was performed by using DL-glutamic acid-1-C^{14}. Of the starting C^{14} radioactivity, 96% was found in $BaCO_3$. In the unpurified reaction mixture containing succinic semialdehyde, 5% of the radioactivity was found.

d. This experiment was done by using DL-glutamic acid-2-C^{14}. Glutamic acid-2-C^{14} solution was prepared as follows: About 1 mg of the glutamic acid-2-C^{14} (1.32 Mc/mM) was dissolved in about 50% ethanol and made up to 10 ml (Solution N). One hundred ml of this Solution N was added to 10 ml of scintillation solution No. 2 and counted, giving 427,000 dpm/ml of Solution N. One ml of Solution N was used for chloramine T oxidation. The total activity of the succinic semialdehyde solution was 491,000 dpm, whereas the specific activity of $BaCO_3$ was 0.6 dpm/mg or 0.6×1.3425 (theoretical yield) = 805.5 dpm, less than 0.2% of the starting radioactivity. This experiment was repeated and gave: total radioactivity before the reaction, 407,000 dpm; after the reaction, in the reaction mixture, 498,000 dpm. Thus, large differences are noticed between the total radioactivity before the reaction and after the reaction. The low count before the reaction seems to come from the low concentration of glutamic acid, which leads to wall absorption.

Various experiments were performed in an effort to overcome this counting difficulty, and a solution to the problem was found in the addition of HCl and more carrier glutamic acid, as shown in the following experiment. This experiment was performed by starting with 1.000 g of glutamic acid, 1.34 g of concentrated HCl, uniformly labeled glutamic acid, and H_2O to 50 ml total volume. The total activity of this solution was 906,000 dpm/total volume. When the degradation was carried out the activities were found as follows: succinic semialdehyde solution, 737,000 dpm/total volume = 81.3% of total activity; $BaCO_3$, 129 dpm/mg = 129×134.25 = 173,000 dpm, or 19.1%.

This experiment was repeated twice, using 100 mg glutamic acid each time, with the following results:

	% of starting C^{14} found	
	$BaCO_3$	reaction mixture
Exp. 7	17.3	78.5
Exp. 8	17	83.5

2. Wolff-Kishner reduction

The reaction mixture from Exp. 8, containing succinic acid semi-aldehyde in solution, was adjusted to pH 8 and transferred to a 500-ml flask containing 2 g of KOH, 13 ml of 85% hydrazine solution, and 65 ml of redistilled diethylene glycol. The solution was refluxed for 3 hours, and water was distilled off until the refluxing temperature reached 180 to 190°C. Refluxing was continued at this temperature for 4 hours. After cooling, the reaction mixture was acidified with 10 N H_2SO_4, and steam-distilled until all steam-volatile acid had been collected. The end point of steam distillation was determined by titrating several successive fractions of the distillate with standardized NaOH solution.

Steam distillate	Volume of distillate (ml)	Alkali consumed (mM)
1st	524	5.36
2nd	500	0.91
3rd	350	2.57
4th	500	2.30

The above data show that 79% (5.36 mM) of butyric acid was distilled in the first 500 ml. After 1 liter of distillate had been collected, another kind of acid seemed to be distilled.

After the distillates had been alkalized, they were concentrated to about 20 ml and washed with chloroform.

The resulting solution was acidified, then butyric acid was extracted with $CHCl_3$ in a continuous-extraction apparatus. Alkaline titration showed that $CHCl_3$ extract contained 3.22 mM of butyric acid. Final purification was achieved by gas chromatography on a Yucon (polyethylene oxide) column. Yield: 180 mg, 30% based on butyric acid.

3. Schmidt reaction of butyric acid

The apparatus used for the decarboxylation step consisted of a 35-ml reaction flask equipped with sweep tube, a scrubber containing 5% $KMnO_4$ in 0.5 N H_2SO_4, and traps containing 2 N carbonate-free NaOH.

Butyric acid (440.5 mg, 5 mM) was placed in the reaction flask, and after the flask was cooled in an ice bath, 3 ml of 100% H_2SO_4 was carefully added, and then 500 mg (7.7 mM) of sodium azide was added, little by little, very carefully, with stirring in the ice bath. As the bath temperature was raised very slowly, the reaction started smoothly at around 10°C. The reaction proceeded between 12° and 18°, and then the flask was heated at 70° for 45 min. A yield of 982 mg of $BaCO_3$ was obtained, equal to 99.5% of theoretical. In order to recover the resulting propylamine, 25 ml of 5 N NaOH was added to the above reaction mixture while cooling with ice until the pH of the mixture was 11 to 12. Then the system was swept with N_2 for 30 minutes with the bath temperature at 90 to 100°C. The propylamine was collected in a cold trap and contained by a large amount of H_2O. In fact, Kjeldahl nitrogen analysis of the distillate showed a concentration of only 6 to 7% of propylamine by weight. Therefore it is suggested that the propylamine should be collected in a 0.2 N H_2SO_4 trap in the future.

4. Oxidation of propylamine

The propylamine formed was oxidized to propionic acid with 5% $KMnO_4$ (pH 12) at room temperature (90 min). Aqueous $FeSO_4$ was added to remove the excess of permanganate. The mixture was acidified to pH 2 and steam distilled. The resulting propionic acid was purified by the same method as butyric acid. The yield was very poor, about 10%.

5. Schmidt reaction of propionic acid

Schmidt reaction of propionic acid was carried out the same way as with butyric acid. Propionic acid (740.8 mg, 10 mM) was placed in the reaction flask. Six ml of concentrated H_2SO_4 was carefully added with ice cooling and stirring, and then 1.0 g of sodium azide was added a little at a time. The bath temperature was raised to 60 to 70° in a period of 50 min. After 30 min at this temperature the system was swept with N_2 for 30 min. Barium carbonate, 1.9931 g, was obtained; theoretical amount, 9.9737 g. The resulting ethylamine was distilled in strong alkaline solution at 50 to 60° C (bath temperature).

6. Oxidation of ethylamine

Ethylamine (225 mg, 5 mM -- that is, 322 mg of 70% solution) was dissolved into 50 ml of 0.2 N H_2SO_4. Fifty ml of 5% $KMnO_4$ was added to the above solution and the solution was made alkaline above pH 8. The flask was tightly stoppered and heated to 90 to 100° C in a bath for 18 min. After the solution was cooled, aq. $FeSO_4$ was added to remove the excess of permanganate. Then the mixture was acidified to pH 2 and the acetic acid was steam distilled. The distillate was adjusted to pH 8 (or above 8) and concentrated to 10 ml. The concentrated solution was washed with ether and then acidified with 2 N H_2SO_4. The acid solution was saturated with salt and extrated with ether. After evaporation of part of the ether, the remaining liquid was purified by gas chromatography and a Yucon column. The yield of acetic acid was 120 mg.

Conclusions

The degradation of glutamic acid to $BaCO_3$ from carbon atom number 1 and butyric acid has been studied and appears to offer a reasonable yield of the products in uncontaminated form. Degradation of butyric acid via the Schmidt reaction gives a good yield of carbon number 5 as $BaCO_3$.

Oxidation of propylamine from the Schmidt reaction on butyric acid does not appear feasible without further work. A possible alternative method might be a chromic acid oxidation of part of the butyric acid under conditions that would give a mixture of propionic and acetic acids. These 3-carbon and 2-carbon fatty acids could then be purified by vapor-phase chromatography and counted separately to give carbon atoms 2, 3, and 4, and carbon atoms 2 and 3. However, no study has yet been made of these possibilities.

THE CHEMICAL EFFECTS OF IONIZING RADIATION ON NUCLEOTIDES

Cyril Ponnamperuma, Richard M. Lemmon, and Edward L. Bennett

The principal interest in the radiation chemistry of nucleic acids and related compounds is derived from the important role these materials play in hereditary processes. The genetic changes caused by ionizing radiation appear to be linked to chemical or structural changes in the nucleic acid molecule.

Previous workers have observed physical effects such as changes in viscosity, optical rotation, rate of diffusion, etc. when nucleic acid solutions were irradiated with x-rays.[1] Scholes and his coworkers[2, 3, 4] demonstrated the increase in amount of nitrogen determined by the Van Slyke method, the liberation of inorganic phosphate, and the ring opening of the bases when aqueous solutions of nucleic acids were subjected to large doses of x-rays in the presence of air.[2, 3, 4] Hems irradiated a dilute solution of adenosine diphosphate in air and showed that the absorbency decreased linearly up to 3×10^5 rads.[5]

In our experiments, a study is being made of the effect of radiation on the nucleotides adenylic acid, guanylic acid, cytidylic acid, and uridylic acid. These compounds uniformly labeled with C^{14} (specific activity 1.0 μC/mg) were obtained from Schwarz Laboratories, Mount Vernon, New York. The compounds were tested for their radiochemical purity by means of paper chromatography. The uridylic acid was the only one in which the radiochemical impurities did not add up to more than 0.1% of the total activity. This nucleotide was therefore chosen as our first target material.

The other nucleotides had impurities amounting to about 5% of the total activity. The latter would therefore have to be purified before irradiation. As no information is available about the purity of the compounds when they were first prepared, it is not possible to say whether the presence of the impurities was due to self-irradiation.

For the irradiation, samples of uridylic acid were vacuum sealed in capillary tubes of 3 mm diameter. The capillaries were then placed in holders in which they could be exposed to Co^{60} γ rays or to the electron beam of a linear accelerator. For irradiation of the solid, 0.1 mg of

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[1] K. A. Stacey and P. Alexander, in Progress in Radiobiology (Oliver and Boyd, Edinburgh, 1956), p. 105.

[2] G. Scholes and J. Weiss, Exptl. Cell Research, Supplement 2, 219 (1952).

[3] G. Scholes and J. Weiss, Experientia 11, 219 (1955).

[4] M. Daniels, G. Scholes, and J. Weiss, Biochem. J. 56, 65 (1954).

[5] G. Hems and M. Eidinoff, Radiation Research 9, 305 (1958).

substance was used; for irradiation of solutions, 5 to 10 λ of 0.1% uridylic acid was used. The conditions for the various experiments are summarized in Table VI.

Table VI

Conditions of irradiation of uridylic acid samples

Experiment	Sample	Method of irradiation	Dose (in 10^6 rads)
1	solid	Linac	121
2	solid	Cobalt-60	120
3	solid	Linac	140
4	0.1% solution	Linac	1.8
5	0.1% solution	Linac	3
6	0.1% solution	Linac	18
7	0.1% solution	Cobalt-60	30

The irradiated material was then chromatographed on Whatman No. 1 paper by using isobutyric-acid–ammonia as solvent.[6] The distribution of activity on the chromatograms was recorded by the automatic chromatogram scanner and with the aid of x-ray film. The chromatograms were also examined for materials absorbing ultraviolet light.

Results

In Experiments 1 and 2 no evidence of radiation decomposition was obtained. In Experiment 3, uridine and uracil were both formed; about 5% decomposition was observed. The chromatograms in Experiments 4 and 5 showed undecomposed uridylic acid. In Experiment 6 some uracil was formed. In Experiment 7 almost complete disintegration of the pyrimidine ring was evident. The area containing about 95% of the total activity on a paper chromatogram of the irradiation products showed no ultraviolet absorption. One of the compounds in this area of the paper may be ribose phosphate. The only spot on the chromatogram that absorbed uv light corresponded to uracil and had an activity of only 1% of the total, indicating that most of the heterocyclic ring was destroyed. Work is now in progress to further identify the disintegration products. A systematic study of the other nucleotides will be made.

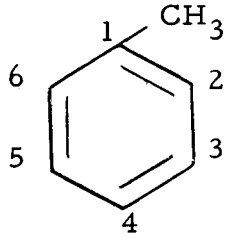
[6] Lofgren, Acta Chem. Scand. 6, 1030 (1952).

EXPERIMENTS ON THE DEGRADATION OF TOLUENE

R. Terrence Mullen and Richard M. Lemmon

The construction of an ion accelerator and its potential use in studying the chemical interaction of accelerated C^{14} ions with organic compounds has been described in a previous report.[1] A degradation of toluene, formed when benzene in the solid state is irradiated with C^{14} ions,[2] has been partially completed. It is hoped that knowledge of the distribution of the C^{14} in the ring of toluene so formed will provide some insight into the mechanism of this reaction in particular, and of such "synthesis" processes in general. It is reasonable to assume that there will indeed be some activity in the ring of toluene produced in this way, since it has been found that the toluene formed by recoiling C^{14} in benzene contains 14% of its activity in the ring.[3]

In this discussion, the carbon atoms of the toluene ring are designated as follows:



The 2 and 6 carbons are equivalent, as are the 3 and 5 carbons. Hence, four values are required for the ring distribution; the 1-carbon, the 2-6, the 3-5, and the 4-carbon.

A method has been investigated that gives the 1-carbon directly and the 2-6 carbons by difference from a 1:1 mixture of $C^{14}O_2$ derived equally from the 1- and the 2-6 carbons. This method was checked with both commercial toluene-1-C^{14} and cyclohexanone-1-C^{14}. Parts of the following scheme (Fig. 7) have been used[4, 5, 6] and checked[5, 6] by other workers in analogous degradation studies of related compounds. The percentages given are the yields obtained by us in these reactions.

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[1] R. T. Mullen, R. M. Lemmon, and F. L. Reynolds, in Bio-Organic Chemistry Quarterly Report, UCRL-8575, Sept. 1958, p. 8.

[2] R. M. Lemmon, F. Mazzetti, F. L. Reynolds, and M. Calvin, J. Am. Chem. Soc. 78, 6414 (1956).

[3] A. P. Wolf, B. Gordon, and C. S. Redvanly, Abstracts of Papers, 131st Meeting of American Chemical Society, Miami, Florida, April 1957.

[4] A. E. H. Kilner, H. S. Turner, and R. J. Warne, Radioisotope Conference, Proceedings of the Second Conference, Oxford, July 19-23, 1954 (Academic Press, Inc., New York, N. Y.) p. 29.

[5] R. B. Loftfield, J. Am. Chem. Soc. 73, 4707 (1951).

[6] J. D. Roberts, D. A. Semenow, H. E. Simmons, Jr., and L. A. Carlsmith, J. Am. Chem. Soc. 78, 601 (1956).

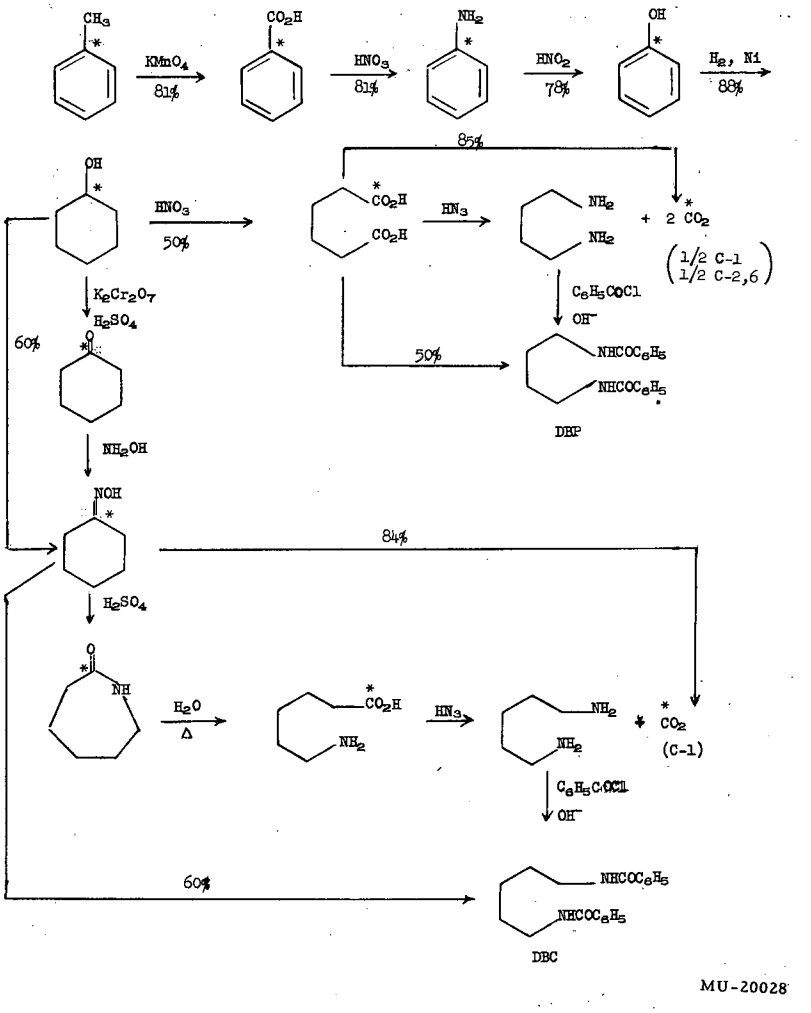


Fig. 7. Toluene degradation scheme.

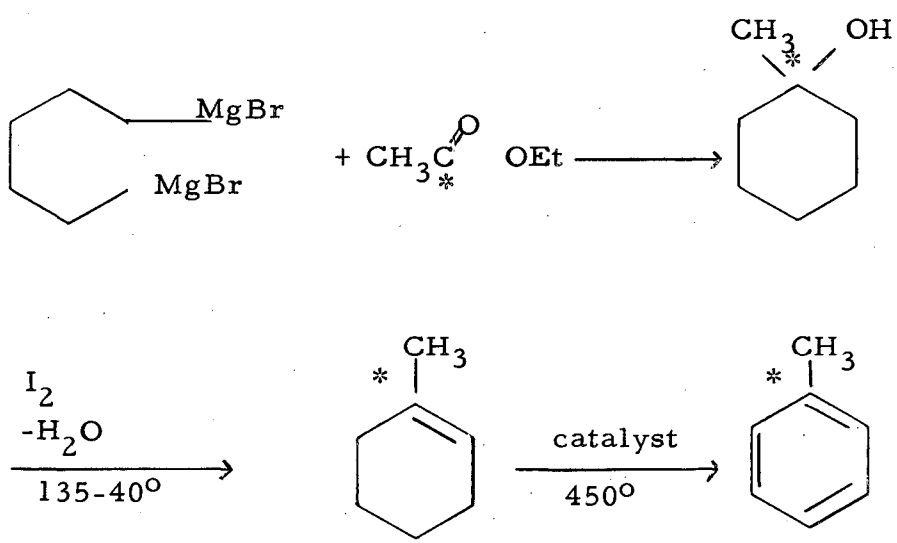
If this series of reactions goes without any rearrangements, the carbon dioxide from the Schmidt reaction on ϵ-aminocaproic acid (ϵACA) is solely from the carbon-1 and should therefore have the same specific activity as the parent compound; furthermore, there should be no activity in the dibenzoylcadaverine (DBC), which contains carbon atoms 2 through 6. Likewise, the Schmidt reaction on adipic acid produces the carbon dioxide, one-half of which comes from carbon-1 and one-half from the carbons 2 and 6. This carbon dioxide should have one-half the molar specific activity of the parent adipic acid. The dibenzoylputrescine (DBP), containing carbons 3, 4, 5, and 6, should have no activity. The data that follow apply to the checks we have made using toluene-1-C^{14} (Table VII) and cyclohexanol-1-C^{14} (Table VIII).

The small amount of activity remaining in the DBC and DBP derivatives made from the toluene-1-C^{14} may be due to one or more of the following possible causes:

1. Radiochemical impurity. This is unlikely even though the toluene contained around 9% radiochemical impurity, as shown by gas-phase chromatography. Simply oxidizing the toluene to benzoic acid removed the bulk of this impurity. It is also unlikely that impurities would persist through the long series of reactions and purifications.

2. Rearrangement. From the essentially perfect check given by the cyclohexanone-1-C^{14}, the portion of the scheme from cyclohexanol on is known to be free from rearrangement. Roberts found no activity in cadaverine made from aniline in a degradation similar in most respects to that shown above.[6] This would leave the benzoic acid-to-aniline reaction as the only possible source of rearrangement, but the Schmidt reaction on substituted benzoic acids proceeds without any nuclear rearrangement.

3. Actual labeling in positions other than the 1-carbon in the ring of the toluene. This is perhaps the most probable cause. It could be due either to rearrangement during the synthesis of the toluene, or to rearrangement of the undiluted high-specific-activity toluene by self-irradiation, possibly proceeding through a symmetrical tropylium ion. The toluene-1-C^{14} used was prepared (commercially) as follows:[7]



[7] A. Murray and D. L. Williams, eds., Organic Syntheses with Isotopes, Part I (Interscience Publishers, Inc., New York, N. Y. 1958), p. 102.

Table VII

Results of the degradation of toluene-1-C^{14}

Compound	Samples counted	Specific activity (cpm/mM	% of molar specific activity of adipic acid (taken as 100%)
Toluene	(standard taken as 140 cpm/mM	14,880	
Benzoic acid	2	13,700	99.0
Aniline	1	13,690	98.9
Phenol	1	13,350	96.5
Cyclohexanol	1	13,320	96.4
Cyclohexanone oxime	3	13,620	98.5
Adipic acid	3	13,820	100
Carbon dioxide-1-carbon	2	14,050	101.7
Carbon dioxide-1- and 2-6 carbons	2	6,790	49.0
DBC	2	231	1.67
DBP	2	179	1.29

Table VIII

Results of the degradation of cyclohexanol-1-C^{14}

Series 1[a]

Compound	Samples counted	Specific activity (cpm/mM)	% of molar specific activity of adipic acid (taken as 100%)
Cyclohexanol	7	107,400	99.8
Cyclohexanone oxime	1	102,000	94.9
Adipic acid	1	107,600	100
Carbon dioxide-1-carbon	2	107,600	100.0
Carbon dioxide-1- and 2-6 carbons	2	52,400	48.7
DBC	not counted[b]		
DBP	2	32.6	0.03

Series 2

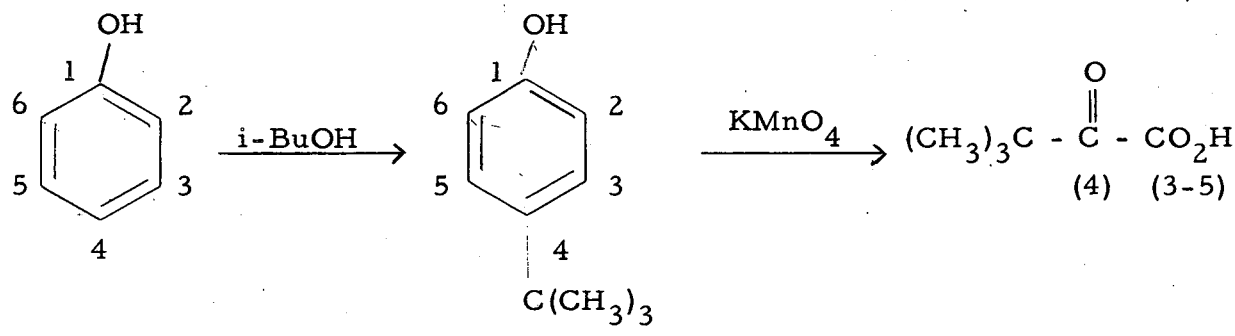
Compound	Samples counted	Specific activity (cpm/mM)	% of molar specific activity of cyclohexanol (taken as 100%)
Cyclohexanol	2	106,300	100
Cyclohexanone oxime	3	107,900	101.2
Carbon dioxide-1-carbon	3	107,200	100.8
DBC	2	40.4	0.04

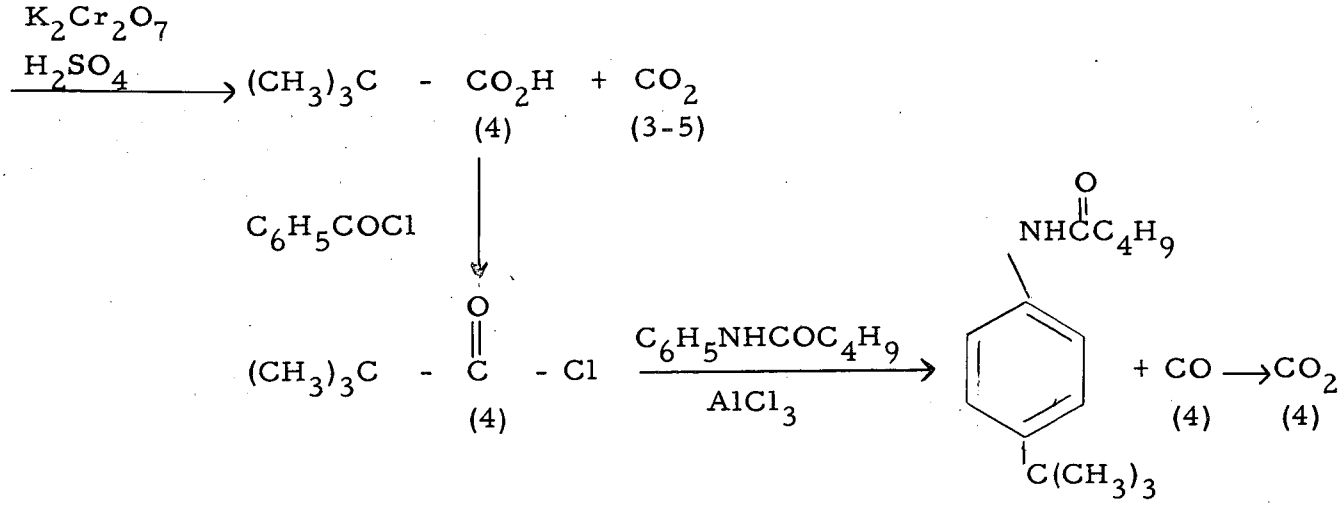
[a]Commercial cyclohexanone-1-C^{14} was hydrogenated and used in these two series.

[b]The dibenzoylcadaverine was contaminated by an active, difficultly separable impurity, believed to be a dipeptide. It occurs only when the Schmidt reaction of ϵ-aminocaproic acid is run in the presence of SO_3.

The last step, the dehydrogenation, is severe enough (450°) that some rearrangement would not be surprising.

It was originally intended in this work to use a series of reactions reported by Reio and Ehrensvard to obtain the isotopic content of the 3-5 and the 4-carbons.[8] These reactions are as follows:





On checking this procedure with phenol-1-C^{14}, prepared from toluene-1-C^{14}, we found repeatedly that activity appeared in the carbon dioxide that was supposedly from the 3-5 carbon of the parent phenol. The specific activity of the carbon dioxide was 19.9%, 17.7%, and 19.7% that of the phenol. Obviously, one or more of the above reactions was not going as written, or else some side reaction was also occurring, rendering the method useless for degradative purposes. There are several possible alternative methods for obtaining the 3-5 and 4-carbon values, and two of these are now being investigated.

[8] L. Reio and G. Ehrensvard, Arkiv Kemi 5, 301 (1953).

EXAMINATION OF A LIQUID SCINTILLATION COUNTING METHOD FOR CARBON DIOXIDE-C^{14}

Fritz H. Woeller and Richard M. Lemmon

The principle of binding $C^{14}O_2$, by means of a quaternary ammonium base, into a form suitable for liquid scintillation counting has been used elsewhere for several years.[1,2,3] The work described in this report has investigated this technique with two objectives: (a) to develop rapid-processing methods for single samples, and (b) to expand the applicability of the assay to $C^{14}O_2$ originating from combusted organic material. These objectives appear to have been satisfactorily realized.

Experimental Procedure

In the preparation of the quaternary ammonium base "hyamine" [(p-diisobutylcresoxyethoxyethyl) -dimethylbenzyl-ammonium hydroxide)] we have followed closely the procedure given by Eisenberg.[2] Essentially, commercial Hyamine 10-X is recrystallized from reagent-grade toluene and then, in methanol solution, treated with silver oxide. The silver chloride precipitate is rapidly removed by centrifugation in order to minimize its re-solution in the newly formed Hyamine. Nevertheless, appreciable amounts of silver chloride do dissolve and must be eliminated by repeated exposure of the preparation to sunlight and by centrifugation of the resultant silver precipitate. The initial recrystallization of the commercial hyamine hydrochloride (Rohm & Haas 10-X; mp about 140°) must be done carefully, since the solubility-vs.-temperature curve for toluene solutions appears to have a steep gradient. In general, the preparation of a reagent of good appearance has become a routine matter.

The technique used in all experiments has consisted of a final direct release of CO_2 into a liquid-scintillation counting vial (20-ml; low potassium glass, Wheaton Glass Co., Millville, New Jersey) containing the basic reagent. The diffusion unit that is used is operated at reduced pressure and it is constructed with wide cross sections to facilitate diffusion. The pressure employed is determined by the vapor pressure of the methanol solvent; this is about 100 mm at room temperature but can be lowered to about 10 mm by the use of an acetone-ice bath. The apparatus for the generation of $C^{14}O_2$ from $BaC^{14}O_3$ is shown in Fig. 8. The release of CO_2 is effected by admitting concentrated sulfuric acid into the generator through a dropping funnel.

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[1] J. M. Passman, N. S. Radin, and J. A. D. Cooper, Anal. Chem. 28, 484 (1956).

[2] F. Eisenberg, Jr., in Liquid Scintillation Counting, C. G. Bell and F. N. Hayes, Editors (Proceedings of a Conference held at Northwestern University, August 20-22, 1957) (Pergamon Press, New York, 1958), p 123.

[3] Technical Bulletin Hydroxide of a Hyamine 10X, January 1960, Packard Instrument Company, Inc., La Grange, Illinois.

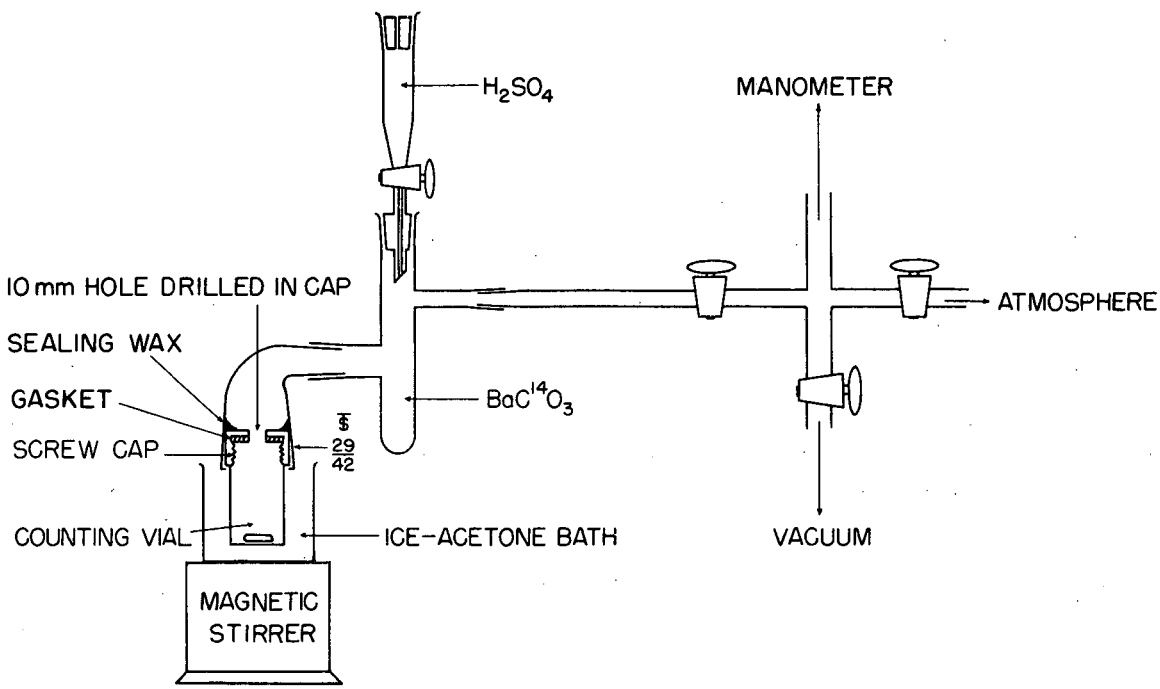

Fig. 8. Apparatus for quantitative transfer of $C^{14}O_2$ (from $BaC^{14}O_3$) into hyamine solutions.

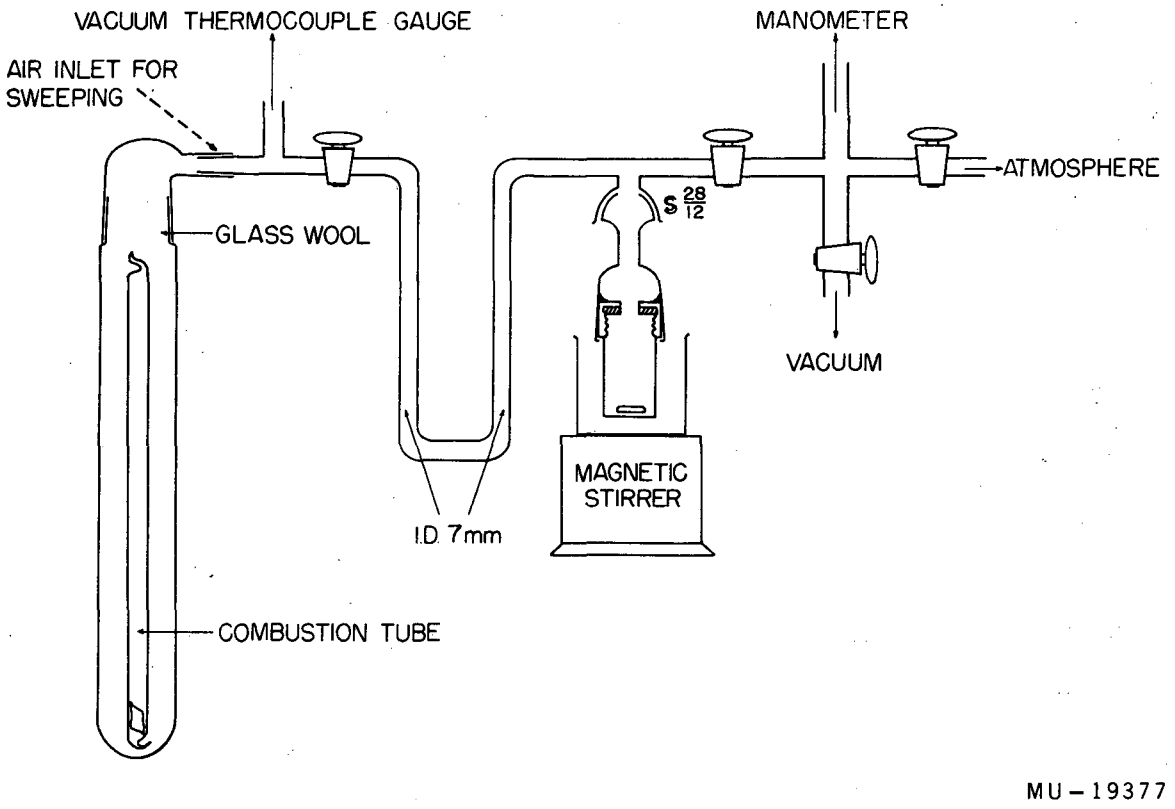

Fig. 9. Apparatus for quantitative transfer of $C^{14}O_2$ (from combusted organic samples) into hyamine solution.

The suitability of the method for $C^{14}O_2$ from a combusted organic sample was tested with benzoic acid-C^{14}. This acid was first combusted by a previously described procedure[4] and the $C^{14}O_2$ transferred in the apparatus shown in Fig. 9. During the first part of the procedure the $C^{14}O_2$ is transferred under high vacuum and frozen into the U tube at -196°. After installation of the adapter with the counting vial (initially a simple glass plug is at this point) the gas is released from the trap by removal of the liquid nitrogen bath.

Agitation of the hyamine solution is provided by a small teflon-coated magnet stirrer. Before the counting vial is detached, atmospheric pressure in the diffusion unit is restored by sweeping air from the most remote point towards the vial region. The total time required for the final diffusion step appeared to be less than 25 minutes in either procedure.

After the vial is detached, the liquid-scintillation solution (toluene, DPO, POPOP) is added to the desired volume and the stirrer is removed.

Results and Discussion

In several sets of experiments with either barium carbonate or combusted benzoic acid, fixed amounts of hyamine reagent were used while the sample sizes were varied by factors of as much as six. A minimum proportion of 1.2 ml of 1 M hyamine solution per mM of CO_2 was used. Passman and his co-workers[1] give the ratio (moles of hyamine to moles of CO_2) needed for quantitative recovery as ≥ 1.1. In our work, there was no detectable trend with respect to the relative excess of base over CO_2.

It appears that, with our equipment, the over-all counting efficiency for $C^{14}O_2$ can be kept above 45% if counting solutions are used that contain a maximum of 35% by volume of the hyamine reagent (equivalent to 3 mM of CO_2 in 10 ml of solution). Higher concentrations of hyamine lead to a sudden sharp drop in efficiency. Furthermore, beyond this concentration limit, a very large increase in the counting rate was noted during the cooling period prior to measurement. This effect would make reproducibility extremely hard to obtain with more concentrated solutions.

A source of difficulty in the procedure is a slightly yellowish discoloration of the reagent. This discoloration becomes more pronounced as the solution stands (over several weeks). In addition, the intensity of the discoloration depends on the type of liquid scintillation solution used. In all cases, the color begins to disappear when CO_2 is introduced. This is accompanied by a moderate increase in counting efficiency. Obviously, results would be variable if appreciable amounts of CO_2 were taken by the scintillation solution in the time between the first and second counts. However, it appears that no measurable change is produced by atmospheric CO_2 during the injection of the internal counting standard into the solution.

We have found that it is desirable to minimize contact between the hyamine solution and the materials below the tin foil in the cap of the Wheaton counting vials. In cases where the tin foil was damaged we have observed a discoloration and, consequently, erratic results.

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[4] I. M. Whittemore, A. E. Ludwigsen, and B. M. Tolbert, in Chemistry Division Quarterly Report, UCRL-3629, Jan. 1957, p. 29.

EFFECTS OF ENVIRONMENTAL COMPLEXITY AND TRAINING ON BRAIN CHEMISTRY

Edward L. Bennett, Hiromi Morimoto, Marie Hebert, and Ann Ohlander

(in collaboration with Professors David Krech and Mark R. Rosenzweig, Department of Psychology, University of California, Berkeley)

In previous Quarterly Reports[1] we have described experiments which demonstrated that the activity of the enzyme cholinesterase (ChE) is a determinant of learning ability in the rat. More recently we have been investigating the possibility that ChE activity is also a dependent variable in the brain and may be influenced by environmental factors. We suggested that increased training and more complex experience would lead to an increase in ChE activity in the cerebral cortex. This hypothesis rests upon three sets of considerations: (a) in previous experiments a possible increase in ChE activity in the brain associated with training has been observed,[2] (b) an increase in ChE as a result of training is consistent with some of the theories of brain biochemistry,[2] and (c) some recent reports from related fields can be interpreted to support the above hypothesis. These latter reports demonstrated that ChE activity is not fixed but instead is responsive to such treatments as changes in acetylcholine (ACh) concentration; decreased stimulation; increased stimulation; and traumatic changes in remote parts of the brain.

To test the hypothesis that increased training and more complex experience can influence brain cholinesterase activity, we separated littermate animals (about 25 days old) into three groups at the time of weaning. The first group, designed as ECT (experimental complexity and training), was kept in a complex environment and trained in standardized test situations.[2] The isolated control animals (IC) were kept in solitary confinement.[2] When available, the third littermate was housed under usual animal colony conditions but received no formal training. These animals served as social controls (SC), and received an intermediate level of experience between the ECT and IC groups of animals.

The rats spent about 80 days in the designated experimental condition. They were then sacrificed and the cortical and subcortical ChE activity determined.[3] Littermate animals were analyzed consecutively.

The number of rats assigned to each experimental condition is summarized in Table IX. The mean ChE values for each of the ECT and IC subgroups are presented in Table X. For every strain, it will be noted that the ECT group is lower than the IC group in cortical ChE activity--contrary to our original hypothesis. On the other hand, for every strain

[1] Edward L. Bennett, Hilda Roderick, Ann Ohlander, Thomas H. Roderick, Hiromi Morimoto, and Charles Longueil, in Chemistry Division Quarterly Report, UCRL-8575, Dec. 1958, p. 34.

[2] Edward L. Bennett, Hiromi Morimoto, Ann Ohlander, Nancy Dye, and Charles Longueil, in Chem. Div. Quarterly Report, UCRL-8698, March 1959, p. 3.

[3] E. L. Bennett, B. Krueckel and A. Halevy, in Chem. Div. Quarterly Report UCRL-3157, Sept. 1955, p. 28.

Table IX

Number of rats in each strain assigned to each experimental condition

Strain	Sex	ECT	SC	IC	Litters sampled
S_1	M	9	6	9	9
S_3[a]	M	20	5	20	16
K	M	11	11	11	11
RDH	M	10	--	10	8
RDL	M	9	--	9	8
RCH	M	7	5	7	7
S_1	F	9	--	9	8
		75	27	75	67

[a]The S_3 rats included two ECT and two IC groups.

Table X

Mean cholinesterase values of experimental and isolated control groups[a]

Strain	N (Pairs)	Cortex[b] ECT	Cortex[b] IC	Subcortical Brain ECT	Subcortical Brain IC	CS Ratio ($\times 10^2$) ECT	CS Ratio ($\times 10^2$) IC
S_1	9	70.1	72.6	170	163	41.5	44.6
S_3	20	59.5	60.1	156	154	38.1	39.0
K	11	60.5	63.1	157	156	38.6	40.4
RDH	10	65.6	66.7	152	150	43.2	44.3
RDL	9	48.0	50.8	135	130	35.5	39.2
RCH	7	71.9	76.2	170	169	42.3	45.0
Over-all	66	61.8	63.8	156	153	39.5	41.5
S_1 female	9	64.8	64.9	172	169	37.7	38.5

[a]All ChE values are in moles ACh $\times 10^{10}$ hydrolyzed per minute per mg of tissue.

[b]ChE activity of the cortex was obtained by averaging the samples of the visual and somesthetic cortical regions.

the ECT group is higher than the IC group in subcortical ChE activity -- in accordance with our hypothesis. These oppositely directed effects are not equally present in all animals. In some pairs the greatest difference between the ECT and IC animals is in the relative decrease of the cortical ChE activity of the ECT littermate, with little difference in the subcortical activity between the two; for other pairs, the greatest difference was in the relative increase of subcortical ChE activity; for still other pairs, the ECT animal showed equivalent decrease of cortical activity and increase of subcortical activity compared with its IC littermate.

These three different patterns can all be expressed in the same way: the ratio of cortical to subcortical ChE in the ECT group is lower than in the corresponding IC group. The ratio of cortical (C) to subcortical (S) ChE for each animal, as a measure of pattern change of ChE in the brain, has been computed. The mean ratios ($\times$ 100) are given in Table X. In every group of animals the CS ratio is lower for the ECT group than for the IC group. The differences in CS ratio between the experimental and control animals were shown to be significant ($p < 0.001$ level of significance) by an analysis of variance. The effects of the experimental treatment were strain-independent.

The generality of the effect of ECT on the CS ratio is clearly shown when each individual ECT animal is compared with its IC littermate control. Each square in Fig. 10 represents the difference between an ECT rat and its IC littermate. In about four-fifths of the comparisons (59 out of the 75 pairs) the IC value is greater than the ECT value. It is also seen that among the 16 negative cases, the deviations tend to be much smaller than among the positive cases.

The effect obtained from the intermediate experience of the SC group is summarized in Table XI. In three of the four strains studied, the cortical ChE activity increased from the ECT to the SC group and increased further from the SC to the IC group. In two strains, the subcortical ChE activity declined from the ECT to the SC group and declined further from the SC to the IC groups. The CS ratio showed an even more general effect, increasing for all four strains from the ECT group to the SC group and again to the IC group. Thus the SC group, which was intermediate between the ECT and IC groups in complexity of environment and which received no formal training, was found to have intermediate ChE values. This indicates that some change in brain chemistry can be obtained even without rewarded problem-solving.

Although the ECT and IC groups were matched in body weight at weaning, at the end of the experiment every IC group was heavier than its corresponding ECT group. The SC group of animals had essentially the same mean weights as the ECT rats. Therefore, it is believed that the greater weight of the IC littermates is due to their relative inactivity. Whatever the cause of the differences in body weight, such differences may be of crucial significance in the present study, since brain weight and ChE activity are negatively correlated. However, the over-all brain weight difference between the IC and ECT groups was only 0.8%. A 13% difference was found in body weight (Table XII). We have concluded that the observed

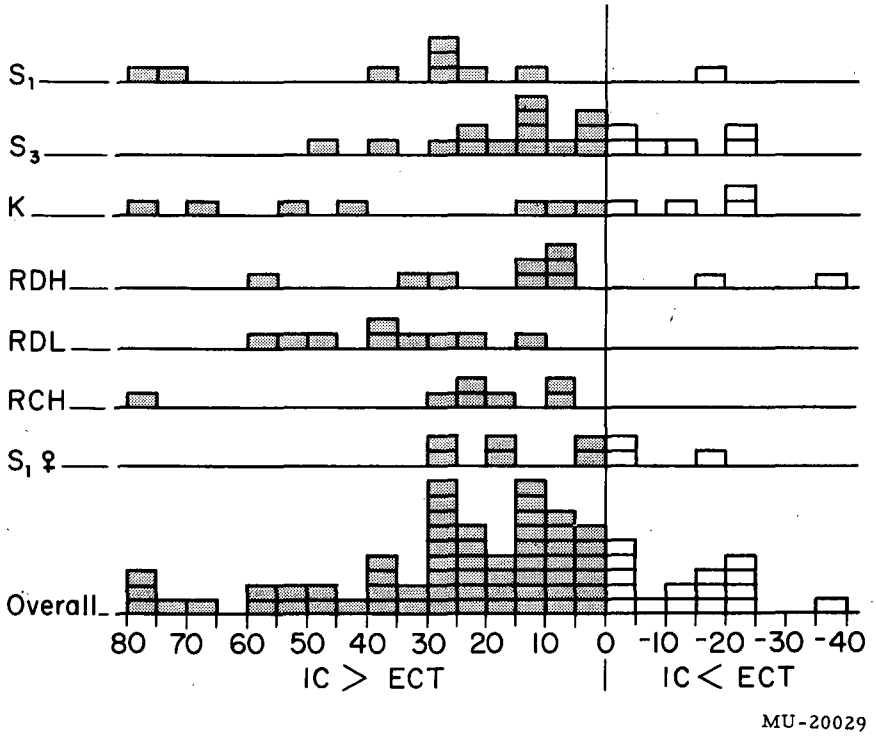

Fig. 10. Differences in CS ratio ($\times 10^2$) between IC and ECT littermates, for six strains.

Table XI

Mean cholinesterase values of experimental, social control, and isolated control groups

Strain	N (triplets)	Cortex ECT	Cortex SC	Cortex IC	Subcortical brain ECT	Subcortical brain SC	Subcortical brain IC	CS ratio ($\times 10^2$) ECT	CS ratio ($\times 10^2$) SC	CS ratio ($\times 10^2$) IC
S_1	6	68.4	70.6	71.9	170.2	169.0	161.8	40.2	41.8	44.4
S_3	5	60.4	60.8	60.4	157.2	165.0	152.2	38.5	39.1	39.7
K	11	60.5	61.8	63.1	157.1	154.1	156.3	38.6	40.1	40.4
RCH	5	72.9	73.5	77.5	172.0	169.0	170.5	42.4	43.6	45.5
Over all	27	64.5	75.7	67.2	162.8	160.5	159.3	39.6	40.9	42.1

Table XII

Mean weight measures of experimental and isolated control groups

Strain	N (pairs)	Body Weight (g) Initial ECT	Body Weight (g) Initial IC	Body Weight (g) Terminal ECT	Body Weight (g) Terminal IC	Brain weight (mg) ECT	Brain weight (mg) IC
S_1	9	53	52	282	325	1612	1602
S_3	20	52	52	264	315	1796	1811
K	11	43	44	336	350	1796	1771
RDH	10	54	54	282	300	1714	1727
RDL	9	59	59	295	359	1852	1879
RCH	7	41	41	254	270	1786	1849
Over-all	66	51	51	284	321	1765	1780
S_1 female	9	45	46	188	197	1549	1529

ChE difference among our groups cannot be attributed to differences in nutritional status. It is most likely, then, that differences in environmental stimulation and training account for the difference in the pattern of distribution of ChE activity between the cortex and the subcortex.

In the experiments described above, the ECT animals were subjected to about 80 days of varied activity and training in order to maximize the possibility of obtaining measurable differences in brain biochemistry between them and the isolated controls. Having now demonstrated that this complex, heterogeneous program has a measurable and remarkably consistent effect on the brain chemistry of the rat, we next decided to investigate which specific aspects of this environmental complexity and training are crucial for producing the biochemical effect. It is highly likely that some of the stimulating conditions or training procedures contributed relatively little to the biochemical effect, while others contributed significantly. Thus, for example, it was found with the SC group that a significant change in CS ratio can occur without the animals' having been subjected to any formal training.

One way in which the conditions of the ECT group differed from the IC and SC groups is in the amount of handling each animal received during the 80 experimental days. Whereas both the IC and SC groups were handled to some extent, the ECT group was handled far more frequently. An experiment was designed to test the possible effects of handling, in interaction with strain, sex, and length of handling. Forty-eight animals (24 littermate pairs) were used in all. Twenty-four animals were of the S_1 strain and 24 were of the S_3 strain. Half the animals of each strain were males and half were females. There were four conditions, each consisting of six animals of each strain, equally divided in sex; (a) a group handled 2 minutes per day for 31 days; (b) a group handled 2 minutes per day for 61 days; (c) and (d) control groups consisting of littermates of groups (a) and (b). This treatment started the day after weaning, as had the treatment of the ECT group. The control littermates were never touched during the experimental period, since all animals lived in wire-bottom cages that did not require cleaning. Immediately after the completion of the handling period, the experimental and their corresponding littermates were sacrificed for chemical analysis.

The results are summarized in Table XIII. Since analysis of variance did not indicate significant interaction effect between handling and length of handling, or handling and sex, these groups are combined in each strain. The handled and unhandled groups do not differ significantly in ChE measures. The lack of statistical significance may reflect the small number of animals in each subgroup or the fact that we tested but two of the six strains. Within the limitations imposed by these qualifications, we conclude for the present that handling per se is not an important factor in the ECT conditions that account for changes in brain chemistry.

Table XIII

Effects of handling on ChE values for two strains of rats

Strain	N (Pairs)	Cortical ChE H	Cortical ChE U	Subcortical ChE H	Subcortical ChE U	CS ratio ($\times 10^2$) H	CS ratio ($\times 10^2$) U	Brain weight (mg) H	Brain weight (mg) U
S_1	12	66.4	68.2	169	170	39.7	41.0	1468	1443
S_3	12	59.4	59.6	161	161	37.4	37.6	1571	1603

In addition to a differential in handling, the ECT animals had a much greater opportunity for sheer activity than the IC animals, and the body-weight data seem consistent with the hypothesis that the ECT animals were more active than the IC rats. Locomotor activity by itself may be responsible for increased neural activity in the brain, and therefore might to some degree account for the observed differences among our experimental groups. To test the possible role which this aspect of the ECT conditions had on brain chemistry, eight pairs of littermate male S_1 rats were separated into experimental and control groups at weaning. The experimental group had free access to activity wheels and averaged more than 100,000 revolutions during the experimental period; the controls were housed in similar cages but without access to activity wheels. At about 105 days of age, the animals were sacrificed for chemical analysis.

The results are summarized in Table XIV. None of the differences shown in the table approaches the 0.05 level of significance. The small differences that do appear are in a direction opposite to the one that would be demanded if running activity were to be assigned any role in determining the ECT effect. The activity animals showed a slightly higher CS ratio than did the control, whereas the ECT animals have consistently shown a lower CS ratio than the IC controls. We have not sampled all the strains in this experiment, and the N is relatively small. Since the differences found are opposite in direction from what one might have expected, and since the brain and sex of the animals used were those which have exhibited large ECT effects, we conclude provisionally that muscular or locomotor activity plays no role in the observed ECT effect.

Table XIV

Effects of activity on brain measures for S_1 strain

Group	N	Cortical ChE	Subcortical ChE	CS Ratio ($\times 10^2$)	Brain weight
Exptl.	8	72.6	174	41.6	1610
Control	8	72.9	180	40.5	1564
Diff.[a]		-0.5	-6	1.1	46

[a]None of these differences reaches the .05 significance level.

The results discussed above appear to provide the first demonstration, to our knowledge, of physical changes in the brain as a consequence of modification of experience. The particular direction of change, in which subcortical ChE activity changed in the expected direction while cortical ChE activity changed in the opposite direction, shows that much research is still needed in order to understand the mechanisms of change. The change in the cortical-subcortical ratio of ChE activity gives a more general expression of the consequences of the experimental treatment than the changes in the cortex and subcortex, considered separately. The change in CS ratio was found for four-fifths of the individual cases, whereas the fall of cortical ChE occurred in only two-thirds of the cases and the rise in subcortical ChE also occurred in only two-thirds of the cases. This suggests that the patterning of neural activity among different regions of the brain may be of prime importance. Different individuals, different strains, and perhaps even different species may reach the same patterning through different particular changes. Perhaps the attempt to characterize the ChE activity of the brain is amiss, and the relative activities of functionally diverse substructures must be taken into account.

The changes found in brain ChE following training and experience are small in absolute size, and the question may be raised whether they could be of functional importance. It seems to be characteristic of the brain that its chemistry is held under rather tight control and does not show the variability of other organs. This control may be exerted because even small changes in brain chemistry could have functional consequences.

Furthermore, localized changes in the brain consequent upon experience may be larger than those we have measured so far. Our tissue samples are relatively gross, and greater changes may be found when we perform analyses in more restricted regions of the brain. Preliminary experiments are in progress to sample and determine the ChE activity of various functional areas of the subcortical brain. We plan to determine the relative magnitudes of chemical changes that occur during learning at various sites and in various types of brain structure.

We have shown that cerebral ChE activity, as well as helping to determine learning behavior, can, in turn, be modified by behavioral and environmental circumstances. Previously we had shown that cerebral ChE is under genetic control.[4] Therefore, the ranges of variation of ChE activity that can be accomplished by environmental and by genetic techniques can be compared. Both sorts of effects are illustrated in Table X. For all male animals, the difference in cortical ChE due to treatment is a change of 3.2%; the largest single difference between ECT and IC groups is found for the RCH strain, where it amounts to 6.0%. On the other hand, the extreme cortical difference due to genetic selection (that between the RDL and RCH strains) amounts to 50%. For subcortical ChE activity, the difference between ECT and IC means is 2.0%; the largest single subcortical difference

[4] T. H. Roderick, E. L. Bennett, and H. Karlsson, in Chemistry Division Quarterly Report, UCRL-3950, Sept. 1957, p. 86; E. L. Bennett, H. Karlsson, Nancy Dye, Ann Ohlander, and T. H. Roderick, in Chemistry Division Quarterly Report, UCRL-8421, June 1958, p. 44.

between ECT and IC is found in the S_1 strain, where it amounts to 4.2%. On the other hand, the extreme subcortical difference due to genetic selection (again between the RDL and RCH strains) reaches 28%. Thus, the variation due to genetic factors is many times as great as that so far produced by extensive environmental manipulation. This conclusion does not hold as strongly for our measure of patterning, the CS ratio. The mean CS ratio difference between the ECT and IC groups is 5.1%; the extreme evnironmental difference is found in the RDL strain, where it reaches 10.4%. The extreme difference in CS ratios due to genetic selection (once again between the RDL and RCH strains) is 17%. Here, then, the range due to genetic effect is less than twice as great as that due to environmental treatment. This provides a further indication that the CS ratio of activity is more sensitive to environmental influences than are the primary ChE measures.

EFFECTS OF D_2O ON DROSOPHILA

Ann M. Hughes

The experiments demonstrating the influence of D_2O on (a) the rate of occurrence of nondisjunction in both males and females and (b) parental sterility and reduction in numbers of offspring (as previously reported[1]) have been repeated, but without duplication of results.

The experiment studying the influence of D_2O on nondisjunction in the female was repeated exactly, using the same stocks and the same laboratory procedure. In the previous experiment, the number of progeny resulting from nondisjunction increased from 0.3% in the controls to 0.6% in the experimentals. In the repeat experiment the occurrence was 0.22% in the experimentals and 0.28% in the controls. Approximately the same number of flies was counted in each experiment (20,000 to 25,000). At present we have no explanation for the discrepancy, but are in the process of doing the same experiment the third time in an attempt to determine which of the results are reproducible.

In a repeat of the male nondisjunction experiment, a different male stock was used. (The females were the same homozygous yellow stock.) Instead of having a marker (the + allele for yellow) on only one arm of the Y chromosome, this stock has markers on both arms. The + allele for yellow (sc^8) is attached to the short arm of the Y, and Stone's bar (B^S) is attached to the long arm. With two such markers it is easily determined whether the entire chromosome is lost or whether it is fragmented. In the earlier experiment (in which we could not differentiate between loss of the entire Y and loss of only the short arm) the percentage of flies showing loss of part or all of the Y was increased from 0.04% in the controls to 0.17% in the treated flies. In the last experiment, similar figures are 0.10% in the controls and 0.08% in the treated. However, if only those flies carrying a fragment of the Y chromosome are considered, the percentage occurrence was increased from 0.025% in the controls to 0.081% in the treated flies. The discrepancy between these two experiments may perhaps be explained in part by the difference in the male stock. Although the $sc^8 \cdot Y$ and the $sc^8 \cdot Y \cdot B^S$ chromosomes were bred into the same yellow stock, it is entirely possible that the autosomal influence could be quite different in the two male stocks. We plan to repeat both experiments, with the $sc^8 \cdot Y$ and with the $sc^8 \cdot Y \cdot B^S$. The interesting information obtained in the last experiment is that it appears that D_2O is causing chromosome breakage rather than nondisjunction and total chromosome loss.

The experiment previously reported on the effect of D_2O on sterility and progeny counts in Canton flies[1] was done with an isogenic stock. The experiment has been repeated twice, with the difference that a nonisogenic stock ($Canton^S$) was used. With respect first to the sterility effects of D_2O, a comparison of the three experiments is given in Table XV.

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[1] Ann M. Hughes, in Bio-Organic Chemistry Quarterly Report, UCRL-8961, Sept. 1959, p. 37.

Table XV

Effect of D_2O on fertility in Canton flies, expressed as the percent of sterile matings

		20% D_2O		
	Control	♂♂	♀♀	♀♀ × ♂♂
Isogenic Canton (Expt. 1)	6	29	55	59
Canton^S (Expt. 2)	6	18	25	40
Canton^S (Expt. 3)	15	7	15	23

In all three experiments, the flies were grown in half-pint bottles. Percentages are based on a total of 45 to 55 matings in each case. There is no explanation at present for the nonreproducibility of Experiments 2 and 3, since the same stock was used in each, and the same laboratory procedure was followed.

With respect to the effects of D_2O on numbers of progeny, the results are shown in Table XVI.

Table XVI

Effect of D_2O on number of progeny per mating in isogenic and Canton^S stock

		20% D_2O		
	Control	♂♂	♀♀	♀♀ × ♂♂
Isogenic Canton (Expt. 1)	27	27	7	6
Canton^S (Expt. 2)	36	39	32	30
Canton^S (Expt. 3)	57	60	31	25

In Experiment 2, the offspring were grown in creamers, because of temporary space limitation. This may have caused crowding of the cultures and prevented their growing to optimum numbers, thus explaining the lack of difference between the various groups in Experiment 2. It is for this reason that Experiment 3 was done, in which the offspring were grown in one-half-pint bottles, as had been done in Experiment 1. Therefore, in this part of

the experiments (progeny counts), the only valid comparison is between Experiments 1 and 3, ignoring Experiment 2. The differences observed between Experiment 1 (isogenic stock) and Experiment 3 (nonisogenic stock) agree with our observations in mice that an inbred strain (C_{57}) is more susceptible to the effects of D_2O than a noninbred strain (Swiss).

The experiment on sterility and progeny counts is being repeated once more, this time with an isogenic Canton stock, as in the first experiment.

COMBINED EFFECTS OF D_2O AND IRRADIATION IN THE PRODUCTION OF RECESSIVE LETHAL MUTATIONS IN DROSOPHILA

Ann M. Hughes and Philip E. Hildreth

Earlier experiments on the influence of D_2O on Drosophila indicated that D_2O increased the rate of occurrence of recessive lethal mutations, although the differences were not statistically significant.[1] The increase in the frequency of recessive lethal mutations caused by irradiation is well known. We have now combined the two treatments to see whether the combined treatment would produce lethal frequencies different from either treatment alone.

Samarkand males were grown on normal or 20% D_2O media. They were then subjected to 1000 r (x-ray) at the rate of 112 r per minute. Following irradiation the standard recessive lethal test[1] was carried out, using y^2Bvw^a females. There was no significant difference in the frequencies of recessive lethals between those grown on normal and on D_2O media.

Since it is believed that the effect of D_2O on sperm (in mice, at least) takes place during meiosis I,[2] the above experiment was repeated, this time utilizing sperm which had been in meiosis at the time of irradiation. Again, there was no difference in the rate of recessive lethal production between the D_2O-treated and the control flies. Apparently D_2O treatment has little influence on the rate of lethal production in irradiated sperm cells.

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[1] Ann M. Hughes, in Bio-Organic Chemistry Quarterly Report, UCRL-8575, Dec. 15, 1958, p. 32

[2] A. M. Hughes, E. L. Bennett, and M. Calvin, Proc. Natl. Acad. Sci. U.S. 45, 581, 1959.

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