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Radiation Laboratory

CHEMISTRY DIVISION QUARTERLY REPORT

March, April, May 1956

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Contents

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CHEMISTRY DIVISION QUARTERLY REPORT

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Radiation Laboratory; Department of Chemistry; and
Donner Laboratory of Biophysics and Medical Physics
University of California, Berkeley, California

June 5, 1956

BIO-ORGANIC CHEMISTRY

M. Calvin, Director
Edited by B. M. Tolbert

STUDIES RELATED TO THE BIOSYNTHESIS OF MORPHINE

Melvin Look and Henry Rapoport

Introduction

Though many schemes[1] had been proposed on the biogenesis of morphine in the opium poppy (Papaver somniferum) no illuminating experimental work was ever done to prove or disprove these schemes. The latter statement is generally true for all the alkaloids. What work was done on the biosynthesis of alkaloids involved the rather questionable technique of "feeding" likely labeled intermediates into a plant and isolating the alkaloids. This technique is objectionable, as the plant may have been forced to produce the alkaloid by a different pathway in order to eliminate the foreign intermediate. Our problem thus reverts to the feeding of labeled carbon dioxide to the opium poppies. Most probably, a morphine molecule with equally labeled carbon atoms will form if $C^{14}O_2$ feeding is continued indefinitely. The success of the problem depends on isolating and identifying labeled intermediates on the pathway to morphine. The use of paper chromatography and radiograms for the separation and identification of labeled intermediates is proposed.

Completely C^{14}-labeled morphine was made previously by biosynthesis[2] for the purpose of physiological and drug-addiction studies. The problem of isolating a small quantity of morphine from opium poppies was recently completed by Van Etten[3] by means of ion-exchange resin. One of our problems is the conversion of completely C^{14}-labeled morphine formed by biosynthesis to morphine-C^{14}-N-methyl-C^{12} (Compound I shown in Fig. 1).

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1 R. Robinson and S. Sugasawa, J. Chem. Soc. 3163 (1931).
V. C. Schöpf, Naturwissenschaften 39, 241 (1952).
K. W. Bentley, "The Chemistry of the Morphine Alkaloids," Oxford University Press, London, 1954, Chapter 28.

2 E. M. K. Geiling et al., J. Am. Pharm. Assoc. 39, 512 (1950).
E. S. Mika, Botan. Gaz. 116, 323 (1955).
A. M. Kuzin and V. I. Merenova, Biokhimiya 19, 616 (1954).

3 E. H. Van Etten et al., Anal. Chem. 28, 867 (1956).

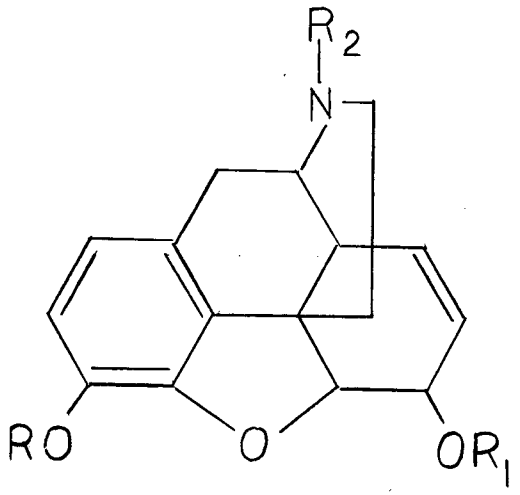

Fig. 1. Morphine and morphine derivatives.

(I) morphine-N-methyl: $R, R_1 = H, R_2 = CH_3$

(II) normorphine: $R, R_1, R_2 = H$

(III) heroin: $R, R_1 = Ac, R_2 = CH_3$

(IV) cyanorheroin: $R, R_1 = Ac, R_2 = CN$

(V) codeine: $R, R_2 = CH_3, R_1 = H$

(VI) N-formylnormorphine: $R, R_1 = H \quad R_2 = CHO$

(VII) O^3, O^6, N-tricarbethoxynormorphine: $R, R_1R_2 = COOC_2H_5$

(VIII) O^3, N-tricarbethoxynormorphine: $R, R_2 = COOC_2H_5, R_1 = H$

(IX) O^3, N-dicarbethoxy, O^6-acetylnormorphine: $R, R_2 = COOC_2H_5, R_1 = Ac$

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Studies with morphine-N-methyl-C^{14} in human metabolism[4] showed that part of the methyl-C^{14} group was eliminated as carbon dioxide in the breath. Further studies of human metabolism necessitate the use of morphine-C^{14}-N-methyl-C^{12}. Morphine was previously converted to normorphine (II, Fig. 1) by von Braun[5] in an over-all yield of about 50%. Development work was conducted to improve the yield, as demethylation in our problem involves the rather precious C^{14}-morphine. Methods were studied also for the conversion of normorphine to morphine-C^{14}-N-methyl-C^{12} in good yield.

Experimental and Results

Development work is being conducted on the separation and spotting of the opium alkaloids on paper chromatograms. Synthetic mixtures and plant extracts are being used. A water-saturated mixture of 5 ml acetic acid and 50 ml n-butanol is showing promise of good separation on unwashed Whatman 4 paper. The alkaloids are spotted in 10- to 20-γ quantities by Dragendorff reagent.[6] Alkaloids form red spots on a pale orange background. There is no reaction with amino acids. The identification of the various spots on paper is being studied. The planned growth of opium poppies under $C^{14}O_2$ is being postponed until seeds arrive from the Treasury Department.

Synthesis of Cyanonorheroin. Variations on the four-step von Braun degradation of morphine to normorphine was undertaken. The experimental data given below were the best of several variations. Morphine was converted to heroin (III) in quantitative yield by refluxing with acetic anhydride for several hours. To a stirred, ice-cold solution of 2.16 g of cyanogen bromide in 40 ml of ethanol-free chloroform was added in 15 min a solution of 6.54 g heroin in 30 ml of chloroform. The greenish solution was stirred for 30 min at ice temperature and 30 min at room temperature. The solution was then heated under reflux for 3 hr. Evaporation of excess reagents and recrystallization of the residue from methanol gave 5.02 g (75%) of cyanonorheroin (IV), mp 232°-235°. Further work-up of the mother liquor indicated a yield of at least 83% cyanonorheroin. Von Braun[5] had reported a yield of 76%.

Normorphine. A suspension of 5.3 g of cyanonorheroin and 24 ml of concentrated hydrochloric acid was swirled on a steam bath for 5 min. Addition of 190 ml of water to the mixture produced an approximately 5% solution of hydrochloric acid. The suspension was heated for 8 hours on the steam bath. The solid went into solution, the solution was filtered and evaporated under reduced pressure to 100 ml. Concentrated ammonium hydroxide was added till the solution was pH 9.2. Normorphine hydrate precipitated and weighed 3.8 g (91%); mp, above 285°, with decomposition. The yield reported by von Braun with a two-step hydrolysis-decarboxylation was 62%.

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4 Elliott, Tolbert, Adler and Anderson, Prox. Soc. Exptl. Biol. Med. 85, 77 (1954).

5 J. von Braun, Ber. 47, 2312 (1914).

6 Bloch, Durrum, and Zweig, "A Manual of Paper Chromatography and Paper Electrophoresis," Academic Press, New York, 1955, p. 245.

Attempted Conversions with Normorphine to Morphine. Considerable trouble is being encountered in the conversion of normorphine to morphine in good yields. Alkylation of normorphine with methyl iodide is unfavorable, as there is appreciable methylation of the phenolic group to codeine (V). Attempts were made to synthesize N-formylnormorphine (VI), which can be reduced with lithium aluminum hydride to morphine. Three methods were tried in a series of attempts to form N-formylnormorphine but there was never any indication that the formyl compound was formed. Normorphine was treated with formic acid, chloral, or methyl formate, and in each case products were isolated that were not compatible with the chemical composition or infrared spectrum of N-formylnormorphine.

Efforts were then directed to form O^3, O^6, N-tricarbethoxynormorphine (VII), which should give morphine with lithium aluminum hydride. Treatment of anhydrous normorphine in pyridine with a benzene solution of ethyl chloroformate gave a complex mixture of products. The products were separable by chromatographing on an alumina column with mixtures of petroleum ether and benzene used for elution. The reaction was abandoned, as the yield of each product was too low to be feasible in radioactive morphine work.

A chloroform suspension of normorphine with a solution of ethyl chloroformate and aqueous potassium hydroxide produced a product in good yield, tentatively identified as O^3, N-dicarbethoxynormorphine (VIII). The sample is compatible with infrared, ultraviolet, and qualitative analysis. Quantitative analysis was unsatisfactory, therefore another sample is being prepared. Further work is being planned to develop this reaction. Acetylation of the O^6 group with acetic anhydride should give O^3, N-dicarbethoxy, O^6-acetylnormorphine (IX), which should give morphine with lithium aluminum hydride.

INTERMEDIATES IN ALGAE METABOLISM

James A. Bassham, S. Alan Barker, and U. Carol Quarck

L-Azaserine (O-diazoacetyl-L-serine), an antibiotic isolated from the culture filtrate of Streptomyces,[1] has been found[2] to inhibit growth of many bacteria. It exhibits little or no activity against representative protozoa or viruses. Alanine, arginine, histidine, and lysine are effective in low concentrations in reversing the inhibition of growth of Kloeckera brevis, while in E. coli significant interference with azaserine activity is obtained with cystine, phenylalanine, tyrosine, and tryptophane.[3]

The synthesis of inosinic acid in a cell-free pigeon liver system is inhibited by azaserine, and this inhibition can be overcome with higher levels of L-glutamine.[4] The actual point of inhibition of inosinic acid synthesis has now been shown[5] to be the conversion of α-N-formyl glycinamide ribotide into α-N-formylglycinamidine ribotide, a reaction requiring the presence of glutamine and adenosine triphosphate.

Effect of Azaserine on Scenedesmus

Two suspensions (total volume, 21 cc), each containing washed Scendesmus cells (packed volume, 0.2 cc) in KH_2PO_4 solution (0.4 cc, 3.2×10^{-6} M) and one of them azaserine (4 mg), were left for 1 hr to achieve steady states with 4% CO_2 in air and with 2 lamps (each 150-w reflector floods) switched on. Each suspension was then allowed to photosynthesize for 5 min with $NaHC^{14}O_3$ solution (0.9 cc, 360 μc) and flushed with air (1 min). The cells were next killed by pouring them into boiling ethanol (88 cc), and were then reextracted with 20% ethanol (100 cc). The total fixation of radioactivity was determined in each case, as well as the proportions present in the 80% and 20% ethanol extracts. The combined extracts of each suspension were concentrated, aliquots calculated to contain 1×10^6 counts per min were applied to washed Whatman No. 4 paper and chromatographed first in phenol-water and then in butanol–propionic acid, no solvent being allowed to run off the paper. When the various radioactivity components detected on the paper were counted, it was found that with azaserine there was a marked lowering of radioactivity of glutamic acid, aspartic acid, serine, alanine, and lipid-phospholipid areas, accompanied by a large increase in the activity of glycolic acid, malic acid, citric acid, α-ketoglutaric acid, sucrose, and glutamine. (See Figs. 2 and 3.) When sprayed with ninhydrin, the chromatograms showed that the increased activity of the

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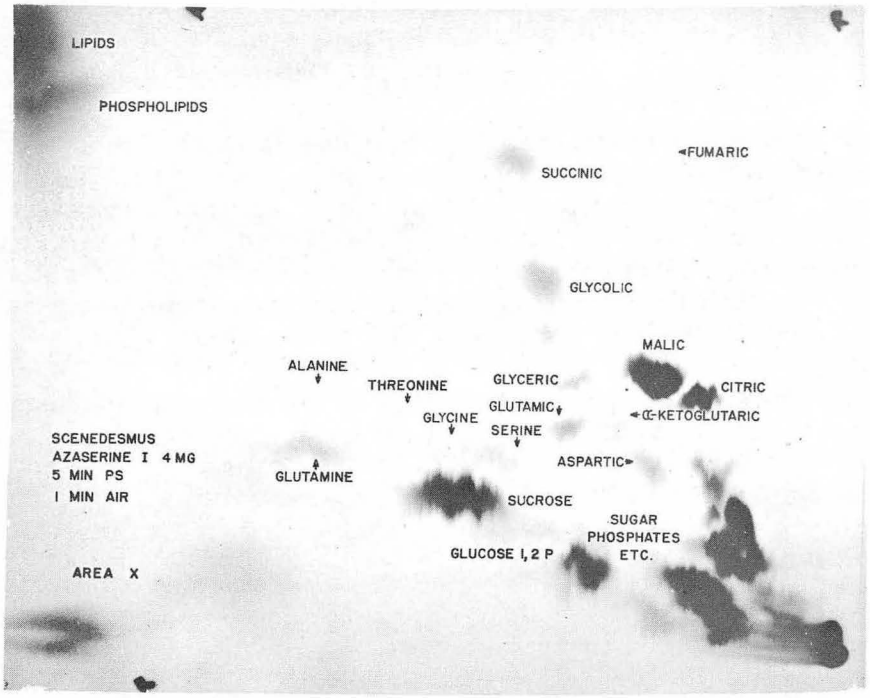
1 Bartz, Elder, Frohardt, Fusari, Haskell, Johannessen, and Ryder, Nature 173, 72 (1954).

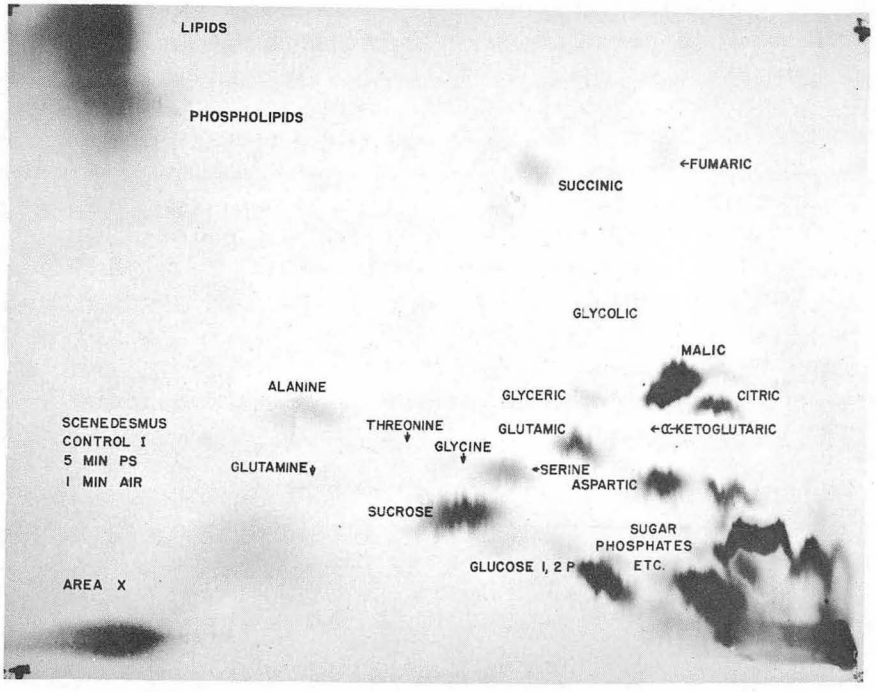
2 Erhlich, Anderson, Coffey, Hillegas, Knudsen, Koepsell, Kohberger, and Oyaas, Nature 173, 72 (1954).

3 H.C. Reilly, Proc. Amer. Assoc. Cancer Research 1, 40 (1954).

4 Hartman, Levenberg, and Buchanan, J. Am. Chem. Soc. 77, 501 (1955).

5 B. Levenberg and J. M. Buchanan, J. Am. Chem. Soc. 78, 504 (1956).





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Figs. 2(above) and 3(below). Effect of azaserine on Scenedesmus photosynthesis.

glutamine was due to the presence of a larger amount of this compound, while the decreased activities of the glutamic acid and aspartic acid spots was due to the presence of smaller amounts of these substances.

Duplicate experiments carried out with 1 mg and 10 mg of azaserine gave similar results.

In a repetition of the 1-mg azaserine experiment an intense photospot light was substituted for one of the reflector floods for 50 min of the 1-hr steady-state period. Under these conditions a bigger proportion of the algae were apparently killed in the presence of azaserine than in its absence, but otherwise the effect of the azaserine was the same as that described for the previous experiments.

Finally, an experiment using 1/10 mg azaserine, performed in the same manner as that described for 4 mg but using algae which had become contaminated with bacteria, produced a rather startling result. In the presence of azaserine, where the bacteria might have been preferentially inhibited, the total fixation was 28.7×10^6 counts per min, while the figure for the control was 18.7×10^6 cpm. The azaserine still produced the same marked effects as in the 1-mg experiment except that only a small amount of glycolic acid could be detected.

In an attempt to reverse the effects of azaserine inhibition with glutamine, an experiment in which one suspension contained 4 mg azaserine and the other both azaserine (4 mg) and glutamine (5 mg) was carried out. The inhibition was only partially reversed. Some increase in the radioactivity of the lipid, phospholipid, area X, and glutamic acid was observed. There was a threefold increase in the radioactivity of the glutamine.

Glutamine and the glutamic acid produced by hydrolysis with 1 N HCl at 100° for two hours were characterized by cochromatography on two-way paper chromatograms, and most of them also by ionophoretic separations. The lipid and phospholipids could not be extracted from the paper with water but were readily extracted with a mixture of petrol ether and ethanol. Area X is an area resembling diphosphopyridine nucleotide in its solubility in phenol-water and extreme insolubility in butanol-propionic acid, but none of this nucleotide could be extracted from the paper with water.

The triose phosphate, pentose phosphate, and glucose cyclic 1,2-phosphate areas were eluted from chromatograms of control and azaserine (10 mg experiment) and treated with purified "Polidase" phosphatase. The products were separated on paper chromatograms, first in phenol-water and then in butanol-propionic acid. The phosphatased triose phosphate area isolated from the azaserine chromatogram showed a component absent from the control, which moved slightly faster than dihydroxy acetone in phenol-water and appreciably faster in butanol-propionic acid. One main additional component was also detected in the phosphatased glucose cyclic phosphate isolated from the azaserine chromatogram. No erythrose was detected in any of these areas, but xylulose was almost certainly present in the phosphatased pentose phosphate areas.

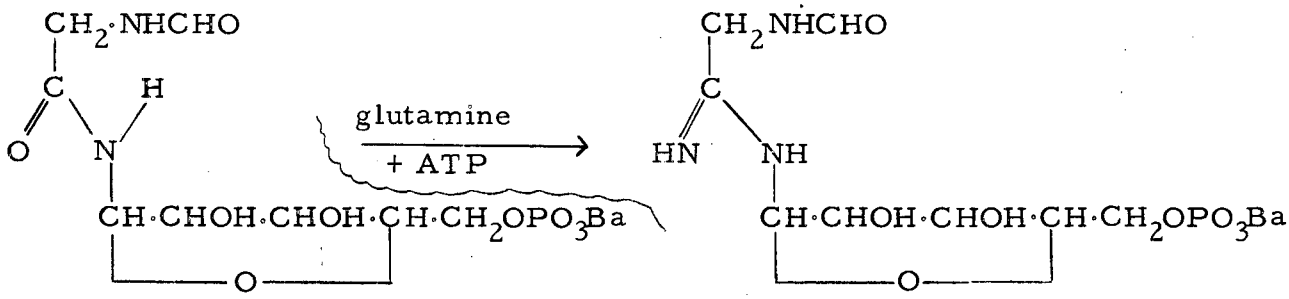
Calvin

Effect of Azaserine on Chlorella

Duplicate experiments with 4 mg azaserine using Chlorella showed that although azaserine caused a marked increase in CO_2 fixation by Chlorella, analysis of aliquots containing equal amounts of radioactivity showed that the radioactivity of most of the metabolites, relative to one another, remained the same.

Discussion

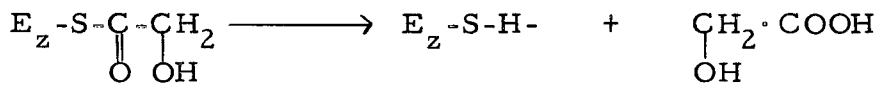
The only enzyme which azaserine has so far been shown[5] to inhibit specifically is that concerned in the amination of α-N-formylglycinamide ribotide (FGAR) to α-N-formylglycinamidine ribotide (FGAM):



FGAR FGAM

With Scenedesmus the azaserine also causes a marked buildup of the acids (e.g., citric, malic, α-ketoglutaric) in the Krebs tricarboxylic acid cycle, and a decrease in many of the amino acids derived therefrom by transamination (glutamic acid, aspartic acid, etc.). Thus one of the main functions of azaserine appears to be the blockage of transamination reactions. The marked decrease in alanine formation (from pyruvic acid) can be ascribed to a similar cause. Since glutamine is a donor of amino groups in many of these reactions it is to be expected that it would accumulate in the presence of azaserine despite the partial inhibition of the formation of glutamic acid (its precursor). It is to be noted, however, that the decrease in glutamic acid is by no means as marked as the decreased formation of aspartic acid in the presence of azaserine.

Glycolic acid, which generally accumulates[6] at low CO_2 pressures, was considerably increased at higher levels of azaserine. This could not be caused by a lower rate of CO_2 fixation since this is, if anything, increased by azaserine during photosynthesis. Wilson and Calvin suggest that glycolic acid arises by a breakdown of a glycolyl-enzyme complex:



glycolic acid

If azaserine speeds such a reaction it may also interfere with the analogous acetyl coenzyme A(CH_3C(=O)-S-CoA), and result in lowering of lipid synthesis in the presence of azaserine.

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

INTERMEDIATES OF THE PHOTOSYNTHETIC CYCLE

James A. Bassham, S. Alan Barker and U. Carol Quarck

Last December, we reported (in UCRL-3240) that chromatography of the phosphatased triose phosphate, produced during 5-minute photosynthesis (1% CO_2) by Scenedesmus followed by flushing with air for 1 minute, showed a radioactive spot which we believed to be dihydroxyacetone but which did not coincide exactly with the authentic compound. It now appears that this spot was not dihydroxyacetone but glyceraldehyde, since exact cochromatography is now obtained with the latter compound. Repeated close examination of this phosphatased triose phosphate area has failed to reveal the presence of any erythrose or erythulose. Neither could these compounds be detected after phosphatasing the ribulose diphosphate or pentose monophosphate areas. The ribulose-xylulose areas obtained from phosphatased pentose monophosphate areas overlap on the normal two-way paper chromatogram. However, upon rechromatography of the rear end of these areas with xylulose, the presence of this sugar was definitely established. The presence of xylulose among the products produced after phosphatasing the ribulose diphosphate area was also confirmed.

Since a tetrose phosphate has been shown[1,2] to be present among the products of the transaldolase-catalyzed reaction between sedoheptulose-7-phosphate and glyceraldehyde-3-phosphate, it was decided to determine whether this tetrose phosphate could be detected among the products of spinach leaf photosynthesis.

A freshly picked spinach leaf, whose stem was immersed in a little culture medium, was left to photosynthesize for 5 min in a stream of 4% CO_2 in air. The CO_2 supply was removed and the surface of the leaf swept with air for 1 min. After a vacuum was created in the cell, $C^{14}O_2$ (generated previously from 5 mg $BaC^{14}O_3$-82 μc/mg) was sucked in and the leaf allowed to photosynthesize for 5 minutes. The spinach leaf was then plunged into boiling 80% ethanol (200 cc). This extract and a 20% ethanol extract (200 cc) were combined, concentrated, and then distributed on ten paper chromatograms. After treatment of all the sugar monophosphate areas with phosphatase and rechromatography, the ribulose-xylulose areas were examined as above and the presence of appreciable quantities of xylulose demonstrated. Again the main triose spot cochromatographed with glyceraldehyde. No erythrose could be detected.

In cooperation with Ning Pon an attempt has been made to detect the unstable intermediate $CH_2O(P) \cdot CHOH \cdot CO \cdot C(OH)(COOH) \cdot CH_2O(P)$, which has been postulated as the first product of carbon dioxide fixation by ribulose diphosphate in the presence of carboxydismutase. The aim was to incubate inactive ribulose diphosphate with $NaHC^{14}O_3$ in the usual digest with carboxydismutase for periods of 10 and 30 min. The reaction was then

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1 B. L. Horecker and P. Z. Smyrniotis, J. Biol. Chem. 212, 811 (1955).

2 Horecker, Smyrniotis, Hiatt, and Marks, J. Biol. Chem. 212, 827 (1955).

Calvin

arrested by pouring the digest into 4 times its volume of cold methanol. Aliquots were applied with cold air onto strips of Whatman No. 4 (unwashed to avoid acid degradation) and then submitted to ionophoresis in 0.12 M $NaHCO_3$ at 600 v for 3 hr, and the papers dried at room temperature. Previous experiments had shown that $NaHC^{14}O_3$ decomposed in propionate buffer, pH 5.2, but was stable and ran as a discrete spot in the 0.12 M $NaHCO_3$. It did, however, leave an appreciable amount (13%) of its activity as another discrete acid-unstable spot on the origin. This phenomenon was not due to using unwhashed paper, since an immobile spot (11%) was also left on washed paper. When a mixture of PGA and $NaHC^{14}O_3$ was separated by ionophoresis in 0.12 M $NaHCO_3$ the PGA ran slightly in front of the $HC^{14}O_3^-$. From previous experience it was expected that the unstable intermediate with three acidic functional groups would run faster than the PGA. No such spot was detected in either the 10-min or 30-min incubation. In both cases a considerable portion of the radioactivity remained on the base line: 10 min fixation, stationary spot 923 x 9.5; mobile activity 2275 x 9.5; 30-min fixation, stationary spot 772 x 9.5; mobile activity 2975 x 9.5 (counts/min).

THE METABOLISM OF THIOCTIC ACID IN ALGAE

Masao Nakazaki, Patricia T. Adams, and James A. Bassham

Since thioctic acid (lipoic acid, protogen) was recognized as a nutrient metabolite essential for the oxidative decarboxylation of α-keto acid by certain bacteria and as a growth factor (protogen) for protozoan, Tetrahymena gel ii, various studies were made in its isolation, structure determination, and synthesis.[1] Meanwhile, this compound was proposed as the possible primary quantum conversion factor in photosynthesis.[2] By use of 6-thioctic-S^{35} acid, the metabolism of thioctic acid in algae was studied in this laboratory,[3] and the conversion of thioctic acid into some kind of lipid was found.

For clearer understanding of the nature of this lipid, some further preliminary studies have been made. It first seemed necessary to obtain the best condition for production of this lipid form of thioctic acid in Chlorella and Scenedesmus, and time reactions in dark and light have been carried out for this purpose. As is seen below, these studies led to the conclusion that in Chlorella, the formation of the alcohol-soluble forms (in 90% alcohol) of thioctic acid (almost entirely the lipid) were very much faster in the light than in the dark, whereas the opposite effect of light was

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1 V. H. Cheldelin, "New and Undetermined Growth Factors," Vitamin III, 575 (1954).
D. M. Greenberg, "Metabolism of Sulfur-Containing Compounds," Chemical Pathway of Metabolism II, 169 (1954).

2 M. Calvin and P. Massini, Experientia 5, 445 (1954).
M. Calvin and J. A. Barltrop, J. Am. Chem. Soc. 74, 6153 (1952).
Barltrop, Hayes, and Calvin, J. Am. Chem. Soc. 76, 4348 (1954).

3 Grisebach, Fuller, and Calvin, Metabolism of Thioctic Acid in Algae, UCRL-3360, April 1956.

observed in Scenedesmus. This fact seems interesting in relation to the earlier observation that there is a difference between Chlorella and Scenedesmus with respect to the acceleration of the Hill reaction by thioctic acid.

For the study of the metabolism of thioctic acid in dark and light reaction the following procedure was employed: A solution of thioctic acid was prepared (3.945 mg/10 ml in 0.067 M KH_2PO_4 and Na_2HPO_4 buffer solution, pH 6.74). The specific activity of the thioctic acid used was 50 μc/mg.

Two 27-ml cell suspensions (KH_2PO_4 10^{-4} mole/l, each containing 0.5 ml of wet packed cells) were aerated 30 min by passing 4% CO_2, one in the dark and one in light, to obtain the steady state. Then 500 λ of thioctic acid solution was added (containing 1.973×10^{-1} mg of thioctic acid).

Aliquots of 5 ml were withdrawn at 5, 10, 20, 50, and 100 min, and each aliquot cell suspension was centrifuged quickly, washed with 5 ml of water, and again centrifuged.

The cells were extracted twice with 5 ml of boiling alcohol (90%) and centrifuged. From this alcoholic extract, an aliquot of 200 λ was withdrawn and counted on a plate. The results of these experiments are shown in Fig. 4.

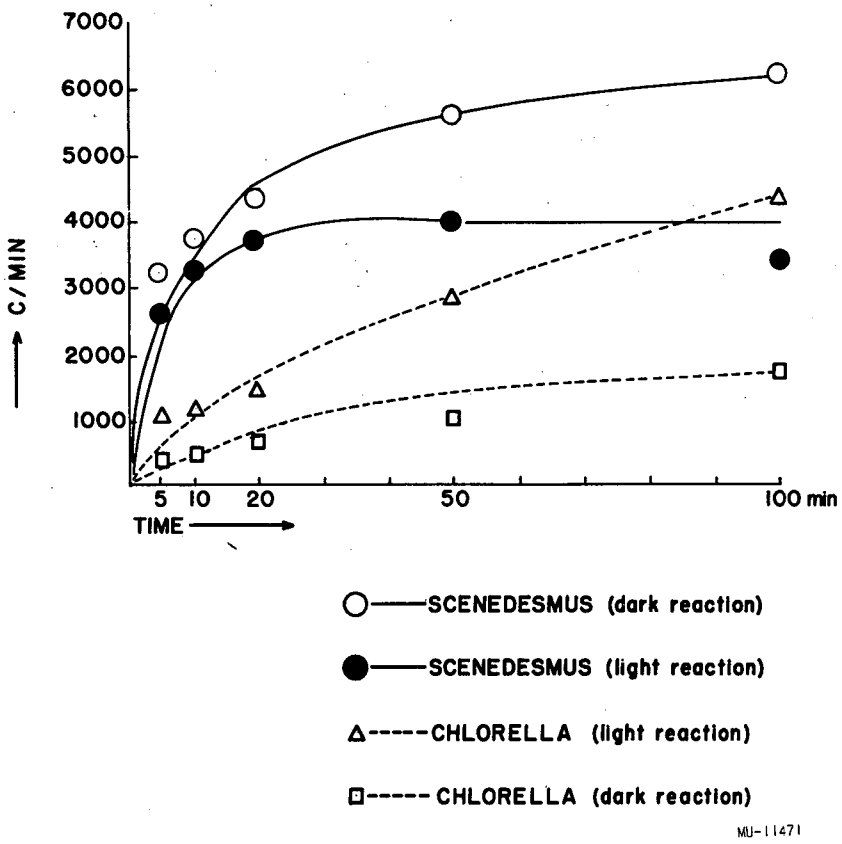

Fig. 4. Uptake of thioctic acid by algae.

THE EFFECT OF AZASERINE ON CARBON DIOXIDE METABOLISM IN ALGAE

Pekka Linko and Osmund Holm-Hansen

As other work in this laboratory indicated striking metabolic changes caused by the addition of azaserine to a suspension of Scenedesmus, it was of interest to extend these observations to a member of the Cyanophyceae. Preliminary experiments indicated that the effect of azaserine on Nostoc muscorum was quite different from that on Scenedesmus, as with Nostoc the activity found in aspartic and glutamic acids increased by a factor of 3 to 5, whereas in Scendesmus the activity found in these acids was markedly reduced. This problem is being studied further.

The effect of azaserine on the in vivo growth of three different algae has also been determined. Results are shown in Table I. It is seen that Nostoc and Scenedesmus are both strongly inhibited by the added azaserine, while apparently there is no effect at all on Chlorella.

Table I

Effect of azaserine on the growth of three different algae

Organism	At start of experiment				After three days' growth	
	Total vol. suspension per flask (ml)	Cell pack vol. per flask (ml)	Dry wt of algae per flask (mg)	Azaserine per flask (mg)*	Cell pack vol. per flask (ml)	Dry wt of algae per flask (mg)
Nostoc	50	0.33	60	0	0.94	116
	50	0.33	60	1.65	0.39	59
	50	0.33	60	6.6	0.34	51
Scenedesmus	50	0.34	75	0	0.71	161
	50	0.34	75	1.73	0.50	108
	50	0.34	75	6.90	0.39	70
Chlorella	50	0.38	54	0	0.65	125
	50	0.38	54	1.8	0.63	126
	50	0.38	54	7.5	0.72	126

* Calculated to yield 1.0 and 4.0 mg azaserine per 0.20 ml centrifuged cell pack of cells.

CHARACTERIZATION OF THE UNKNOWN COMPOUND FORMED DURING PHOTOSYNTHESIS IN BLUE-GREEN ALGAE

Pekka Linko and Osmund Holm-Hansen

Previous quarterly reports have dealt with the experimental conditions necessary for the formation of an unknown compound which occurs during photosynthesis by blue-green algae, and with the isolation of a small amount of this substance by means of ion-exchange resins. This compound has now been characterized, as described briefly below, and found to be citrulline.

In addition to the positive ninhydrin reaction, the unknown was found to have a positive reaction with p-dimethylaminobenzaldehyde, a yellow color developing which is typical for the ureide group (-NH-CO-NH_2). The unknown was then cochromatographed with authentic citrulline, and the spots representing the unknown (C^{14}-active) and the citrulline (C^{14}-inactive) coincided exactly, even to the very uneven outline of the spots. Other experiments confirming the identity of the unknown as citrulline were as follows:

(a) Deamination by nitrous acid caused a loss of activity of 67% on the average (lost as free CO_2). As ninhydrin treatment caused the loss of only 2% to 3% of the activity in CO_2, it seems that most, if not all, the activity is located in the carbamyl group.

(b) Upon acid hydrolysis with 1 N and 6 N HCl, 40% and 60% of the citrulline, respectively, were decomposed to inactive ornithine.

(c) Upon acetylation of the unknown, recovery of the product, and hydrolysis with 1 N HCl, three active compounds were formed (one being the original unknown) as well as a large amount of inactive ornithine.

(d) Hydrogenation with Adams PtO_2 catalyst for 12 hours caused the formation of a compound (with 35% of the activity) which is found above valine on a two-dimensional chromatogram. Citrulline has been treated similarly, and a ninhydrin-positive compound formed which apparently is identical with that formed with the unknown. The proof of this point must still be established.

Attention is now being focused on the time sequence of the formation of citrulline in Nostoc muscorum, as well as its formation both in the light and in the dark.

Calvin

INTERMEDIARY OXIDATIVE METABOLISM

B. M. Tolbert and Martha Kirk

The sulfonamide derivatives, 1-butyl-3-p-tolylsulfonylurea, called "Orinase" and manufactured by the Upjohn Company, and N'-(n-butylcarbamyl)-sulfanilamide, called "BZ-55" and manufactured by the Eli Lilly Company, have been shown to lower blood sugar in normal rats, dogs, rabbits and humans. Further, orally administered Orinase increases liver glycogen without changing muscle glycogen in fasting rats, whereas subcutaneous insulin raises muscle glycogen, but makes no consistent change in the level of liver glycogen.[1]

The mechanism of the hypoglycemic action of the sulfonamides remains to be clarified. Some favor the concept of an "insulin-sparing effect" or insulase inhibition. Others suggest a suppression of glucagon activity. Since anything that affects blood sugar levels must also affect over-all glucose metabolism, we feel that a comparison of the effects of insulin, Orinase, and BZ-55 on the $C^{14}O_2$ excretion patterns for various metabolic intermediates--such as glucose-C^{14}_6, acetate-2-C^{14}, lactic acid, and succinic acid--in normal and alloxan-diabetic rats may give fundamental information on intermediary metabolism as well as some clues on the mechanism of the action of these compounds which change blood sugar levels.

Instrumentation used in determining $C^{14}O_2$ excretion patterns has been described previously.[2] For this study normal Long-Evans rats fasted 18 to 20 hours were given Orinase or BZ-55 (1 g/kg) by stomach tube, or insulin (0.5 unit/kg) by intraperitoneal injection. One and one-half hours later each was injected intraperitoneally with 1 mg glucose-C^{14}_6 containing 10 μc C^{14}, and the $C^{14}O_2$ excretion rates were determined for the next 2 hours. Results are reported for rats with no treatment, insulin, and Orinase. Work on the effect of BZ-55, time after administration of the drug when change in metabolism is at the maximum, and effect on alloxan-diabetic and glucose-loaded rats is in progress. Results are summarized in Table II and Fig. 5. Each curve is the average of a number of rats. These sulfonamide derivatives, as well as insulin, do increase the amount of $C^{14}O_2$ excreted and apparently increase the rate of oxidation, since blood sugar levels are not depressed enough to account for the increased $C^{14}O_2$ excretion on the basis of changes in pool size alone.

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[1] I. Arthur Mirsky, Daniel Diengott, Harry Dolger, Sci. 123, 583 (1956). William L. Miller, Jr., William E. Dulin, Sci. 123, 584 (1956). Lawrence W. Kinsell, Frederick R. Brown, Jr., Roger W. Friskey and George D. Michaels, Sci. 123, 585 (1956). M. Vaughn, Sci. 123, 885 (1956).

[2] B. M. Tolbert, Martha Kirk and E. M. Baker, UCRL-2941, August 10, 1955.

Table II

Effects of insulin and Orinase on the excretion of $C^{14}O_2$ after the injection of glucose-C^{14}_6

Minutes after injection of C^{14}	Σ % of C^{14} as $C^{14}O_2$ [a]					Time of maximum specific activity
	0-20	40	60	90	120	
No treatment	.94	5.14	11.15	20.52	27.85	60 min
Insulin	2.87	9.79	15.88	22.69	27.02	40 min
Orinase	2.54	11.07	19.98	30.18	36.80	41 min

[a] Average of 3 to 5 animals for each value.

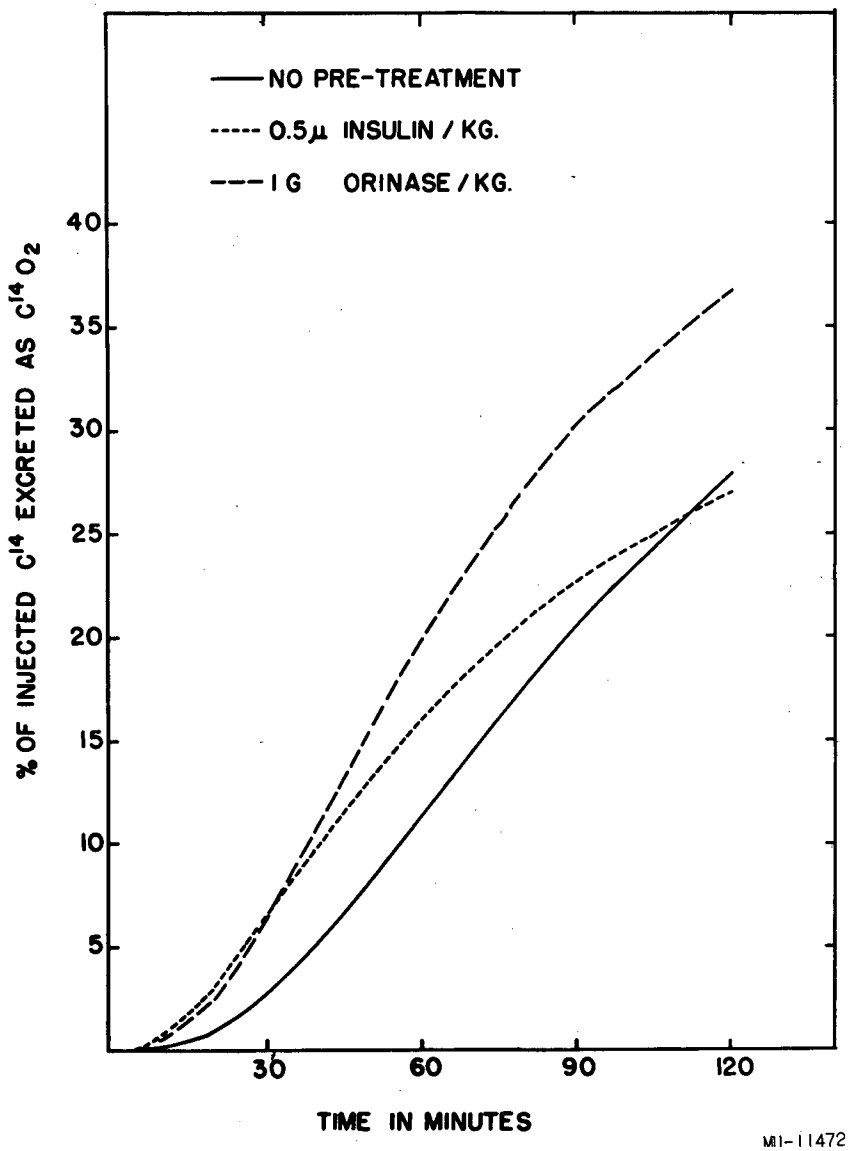

Fig. 5. Cumulative excretion of $C^{14}O_2$ after injection of glucose-C^{14}_6.

EFFECTS OF INHIBITORS ON BRAIN CHOLINESTERASE LEVELS

Edward L. Bennett and Ruth Deane

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

In previous reports, we have presented evidence for a correlation between hypothesis preferences in the rat and the level of cholinesterase activity in the cerebral cortex. In addition, a rationale for the observed correlations has been presented. We are now interested in modifying the level of cholinesterase in the cortex and determining the effects of such changes upon the maze behavior of the rat. (The effect of one drug--sodium pentobarbitol--with a known effect upon the acetyl choline-cholinesterase system has already been studied and reported.[1])

Studies of the inhibition of cholinesterase activity of the rat's cortex and brain by methyl parathion (MPT) were made both to establish toxicity and to determine the inhibition of cholinesterase activity and its subsequent recovery with time. The results of the first experiment, summarized in Table III, indicated that a single dose of 2.4 mg/kg did not produce a significant lasting inhibition of cholinesterase activity of either the brain or the cortex. Subsequently, a second experiment was carried out, in which the animals were given repeated daily injections of 1.2 mg of MPT, and were sacrificed 2 to 2-1/2 hours after the last injection (Table IV). Little or no cumulative effect was observed, and this compound is not believed to be satisfactory for our experiments.

Subsequently we have made preliminary experiments with diisopropyl fluorophosphate (DFP). A marked inhibition of brain and cortex cholinesterase activity was found at the dosage level of 1 to 2 mg/kg. The inhibition was significant after 4 days at the higher dosage level. The results are summarized in Table V. It is expected that preliminary behavioral tests will be started soon.

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[1] Mark R. Rosenzweig, David Krech, and Edward L. Bennett, Science 123, 371 (1956).

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Table III

Effect of methyl parathion upon the cholinesterase activity in the cortex of the male rat*

Time after administration	Range of ChE activity	Average	No. of animals
Control	63-85	72	9
1 to 2 hours	32-66	50	8
1 day	65-78	70	7
2 days	58-78	68	6
3 days	54-71	65	3
6 days	56-74	67	3

* Methyl parathion (2.4 mg/kg) was administered by intraperitoneal injection to 80-day-old male rats. The rats were sacrificed at the indicated times after injection and the "S" area of the cortex was analyzed for cholinesterase activity. The results are expressed in terms of moles of acetyl choline x 10^{10} hydrolyzed/min/mg of tissue under our standardized assay conditions.

Table IV

Cumulative effect of methyl parathion upon the cholinesterase activity in the brain and cortex of the male rat*

Days of administration (days)	Cortex			Brain		
	Range of ChE activity	Average	No. of animals	Range of ChE activity	Average	No. of animals
Control	56-74	64	5	145-161	152	3
1	44-71	55	3	130-134	132	2
2	54-65	58	3	125-148	134	3
3	49-62	56	3	118-140	129	2
4	46-62	47	3	110-149	135	2
5	39-47	43	2			

* 1.2 mg/kg of MPT in C_2H_5OH was intraperitoneally injected in 85-day-old rats daily for the indicated number of days. The animals were sacrificed 2 to 2.5 hr after the last injection and the cortex (S section) and the remainder of the brain with the cortex removed were analyzed for cholinesterase (ChE) activity.

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Table V

Effect of diisopropyl fluorophosphate (DFP) upon the cholinesterase activity of male rat cortex and brain[a]

Time after administration	Cortex ChE activity	Brain ChE activity
Control	68 (average)	152 (average)
2 hours	8; 11	15; 22
2 hours[b]	32; 40	57; 72
1 day	7; 11; 13	17; 25; 25
4 days	19; 16	50; 41

[a] 80-day-old male rats were intraperitoneally injected with 2 mg/kg of DFP and sacrificed at the indicated time. ChE activity was determined in the cortex (S section) and the remainder of the brain with the cortex removed.

[b] 1 mg/kg of DFP was used.

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STUDIES ON NUCLEIC ACID METABOLISM

A. The Incorporation of Adenine-2-C^{14} and Adenine-4,6-C^{14} in C_{57} Mice

Edward L. Bennett, Hilda Karlsson, and Ruth Deane

For reasons discussed in previous quarterly reports, a comparison is being made between the metabolism of adenine-2-C^{14}, adenine-4,6-C^{14} and adenine-8-C^{14}. The results of the first experiment, which have been reported, indicated no large or consistent differences in the specific activity of adenine in 5-AMP, RNA-adenine or guanine, or DNA-adenine or guanine from 1 to 28 days after administration of adenine-8-C^{14} or adenine-4,6-C^{14}. The results of this first experiment indicated a possible difference between adenine-2-C^{14} and adenine-4,6-C^{14}, the difference becoming larger with time. Accordingly, a second experiment has been carried out in which six male C_{57} mice were injected with adenine-4,6-C^{14} and six with adenine-2-C^{14}. Three animals from each group (litter mates) were sacrificed 24 hours after intraperitoneal injection of the adenine, and the other three animals from each group were sacrificed 15 days after adenine administration.

In the previous quarterly report, the specific activities of the 5-AMP from the two series of mice were presented. The analyses for RNA-adenine have been completed, and the preliminary results are presented in Table VI. No significant differences have been observed between the specific activities of the RNA-adenine after adenine-2-C^{14} or adenine-4,6-C^{14} administration. This observation is consistent with the data previously reported for the 5-AMP specific activities.

Table VI

Specific activity (dis/μg) of RNA-adenine of mice injected with adenine-2-C^{14} and adenine-4,6-C^{14}*

Time after Injection (days)	Tissue							
	Small intestine		Large intestine		Liver		Carcass	
	Ad-4,6-C^{14}	Ad-2-C^{14}	Ad-4,6-C^{14}	Ad-2-C^{14}	Ad-4,6-C^{14}	Ad-2-C^{14}	Ad-4,6-C^{14}	Ad-2-C^{14}
1	645	687	821	894	255	313	224	193
	649	698	942	901	355	417	227	204
	536	600	624	868	386	378	199	198
Average	610	662	796	887	365	369	219	198
15	20.7	24.3	30.3	32.4	160	140	146	165
	28.4	23.7	39.4	27.4	157	182	143	125
	30.6	23.2	22.9	31.9	172	193	105	108
Average	26.6	23.7	30.9	30.6	163	172	131	133

* Male C_{57} mice, age 3 months, weight about 25 g, were administered 1.1 mg of adenine-4,6-C^{14} or adenine-2-C^{14}, specific activity, 2.0×10^4 dis/μg.

B. The Utilization of 4-Amino-5-Imidazole Carboxamide-2-C^{14} in C_{57} Mice

Edward L. Bennett and Hilda Karlsson

Preliminary investigations have been initiated on the utilization of 4-amino-5-imidazole carboxamide-2-C^{14} (AIC) in C_{57} mice, by the general methods that we have used for our studies with adenine-C^{14}. We are particularly interested in obtaining a comparison of the nucleotide and nucleic acid specific activities in several tissues and at several time intervals after injection of the AIC, and in making comparisons with similar data obtained with adenine-C^{14}. These first experiments are designed primarily to test methods and to determine the approximate degree of utilization of AIC under our conditions. Results are all tentative pending further check of the purity of the AIC and the results of duplicate experiments.

Two 27-g C_{57} male mice (age 5 months) were injected with 4-amino-5-imidazole carboxamide-2-C^{14} and sacrificed 1 and 3 days later. The mouse sacrificed at 24 hours was given 1.2 mg of AIC containing 4.8×10^6 dis/min, while the mouse sacrificed at 3 days was given 1.9 mg containing 7.7×10^6 dis/min. The specific activity data that are presented have been normalized on the basis of the larger dose.

Table VII gives the approximate percentage distribution of the radioactivity in several tissues and the different nucleic acid fractions. The amount incorporated into the different tissues fractions at 24 hours ranged from 20% to 50% of that found after administration of adenine-C^{14}.

Table VIII presents the data obtained for the specific activities of the 5-AMP, RNA, and DNA adenine 1 and 3 days after the administration of AIC. The low incorporation of the AIC into the DNA of the carcass and the apparently high incorporation into the 5-AMP of the kidney are to be noted. For the other tissues and fractions the specific activities ranged from 15% to 50% of these that would be obtained with adenine under similar conditions. The RNA and DNA-guanine specific activities are also being determined.

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Table VII

Distribution of carbon-14 after administration of 4-amino-5-imidazole carboxamide-2-C^{14} to C_{57} male mice

	Tissue											
	Small intestine		Large intestine		Liver		Carcass		Kidney		Total	
Fraction	1 day	3 days	1 day	3 days	1 day	3 days	1 day	3 days	1 day	3 days	1 day	3 days
Cold TCA fraction	1.4	0.3	0.3	0.14	0.8	0.6	2.6	1.5	0.3	0.14	5.4	2.7
RNA Fraction	0.7	0.26	0.2	0.08	0.4	0.3	0.5	0.4	0.06	0.07	1.9	1.1
DNA Fraction	0.2	0.11	0.05	~0.03	<0.01	<0.01	0.13	0.11	<0.01	<0.01	0.4	0.27
Urine (24 hours)	62%											
Urine (7 hours)		63%										
Respiratory CO_2		2.5%										

Table VIII

Specific activities of 5-AMP-adenine, RNA-adenine, and DNA-adenine after administration of 4-amino-5-imidazole carboxamide[a]

	Tissue									
	Small intestine		Large intestine		Liver		Carcass		Kidney	
Fraction	1 day	3 days	1 day	3 days	1 day	3 days	1 day	3 days	1 day	3 days
5-AMP-Adenine	230	30	120	72	71	40	27.6	24.2	710	39
RNA-Adenine	77	14	83	45	31	27	42	21	51	44
DNA-Adenine	26	7.7	23	18	< 2	2.4	1.4	0.9	< 1	1

[a] Results are expressed in terms of dis/min/μg adenine. Calculated on the basis of administering 1.9 mg of 4-amino-5-imidazole carboxamide-2-C^{14} containing 7.7×10^6 dis/min ⇌ 4050 dis/μg AIC ⇌ 3180 dis/μg equivalent of adenine.

THE EFFECT OF POLIOMYELITIS INFECTION ON THE METABOLISM OF SOME ORGANIC COMPOUNDS IN MICE

Ann M. Hughes, Benjamin V. Siegel, and B. M. Tolbert

The respiratory metabolism of glucose and acetate by poliomyelitis-infected mice has been previously reported.[1] The program has been expanded to include other substrates more specifically involved in brain metabolism, which therefore might be expected to reflect changes induced by the virus infection.

The compounds used (and the dosage per animal) were: formate-C^{14}, 1 mg (~4 μc); L-methionine-methyl-C^{14}, 1 mg (~4 μc); DL-glutamic-1-C^{14} acid, 0.3 mg (~3 μc). The apparatus used was that previously described for the respiratory metabolism of small animals. Webster-strain white mice, 4 to 5 weeks of age, were inoculated intracerebrally with 0.03 cc of a suspension containing 4 LD_{50} (50% lethal dose) of mouse-adapted MEF_1 (Type 2) poliomyelitis virus. Beginning on the fourth day postinoculation, the following groups of animals were used for the metabolic studies:

C: control animals;

O: infected animals, but showing no sign of paralysis;

±: infected animals, showing increased irritability and loss of equilibrium, but no definite paralysis;

+: infected animals, showing definite paralysis of at least one limb.

The results obtained are shown in Figs. 6 through 11. As can be seen from Figs. 6 and 7, there is no difference between the various groups in the total metabolism of sodium formate. However, there is a marked difference in the early rate of metabolism of formate (Fig. 7) between those showing symptoms of paralysis (+ and ±) and those appearing normal (C and O). This suggests the possibility that there is a change in the ability of the brain of the infected animal to oxidize formate--a change that is masked in the long-term experiment when the formate has been taken into the general intermediary metabolism pools.

There is no consistent change in the metabolism of methionine (Figs. 8 and 9).

The metabolism of glutamic acid is not affected by the piliomyelitis infection (Figs. 10 and 11). However, an interesting observation is revealed by an examination of these curves. Figure 11 shows that by the end of 3 hr the excretion of $C^{14}O_2$ is negligible, while Fig. 10 indicates that at the end of that time (and even over a 5-hour total) only about 50% of the injected dose has been decarboxylated. Since the injected compound was

[1] Benjamin V. Siegel and Ann M. Hughes, Glucose Oxidation by Normal and Virus-Infected Mice, UCRL-3063, July 1955;
Benjamin V. Siegel, Ann M. Hughes, and Bert M. Tolbert, UCRL-3316, in press.

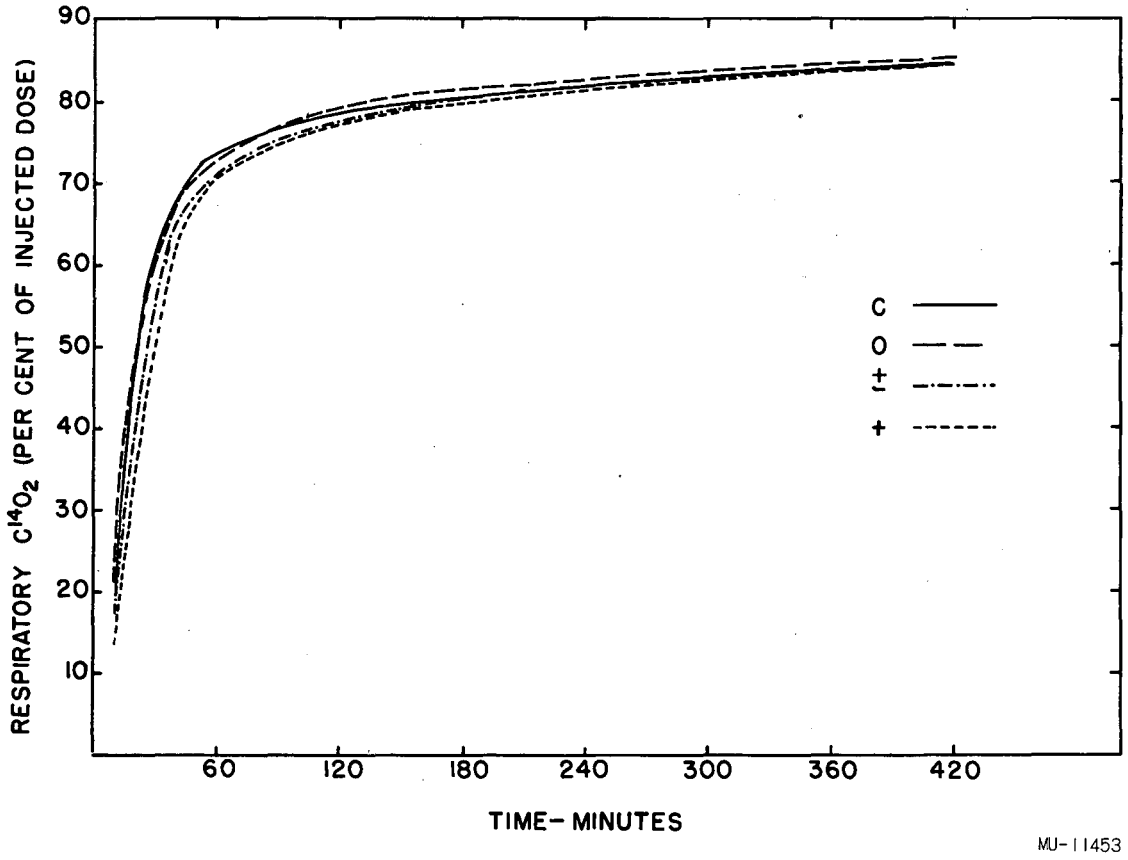

Fig. 6. Cumulative excretion of $C^{14}O_2$ in expired air over a period of 420 min following injection of formate-C^{14}.

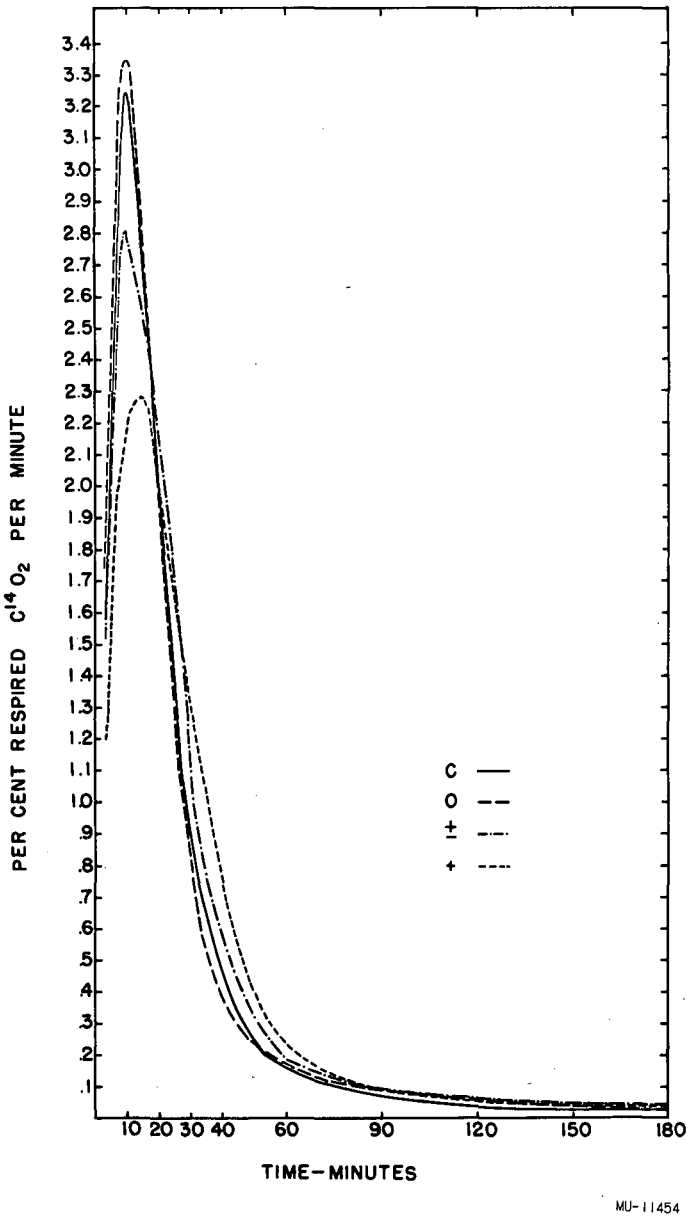

Fig. 7. Rate of excretion of $C^{14}O_2$ in expired air for a period of 180 min following injection of formate-C^{14}.

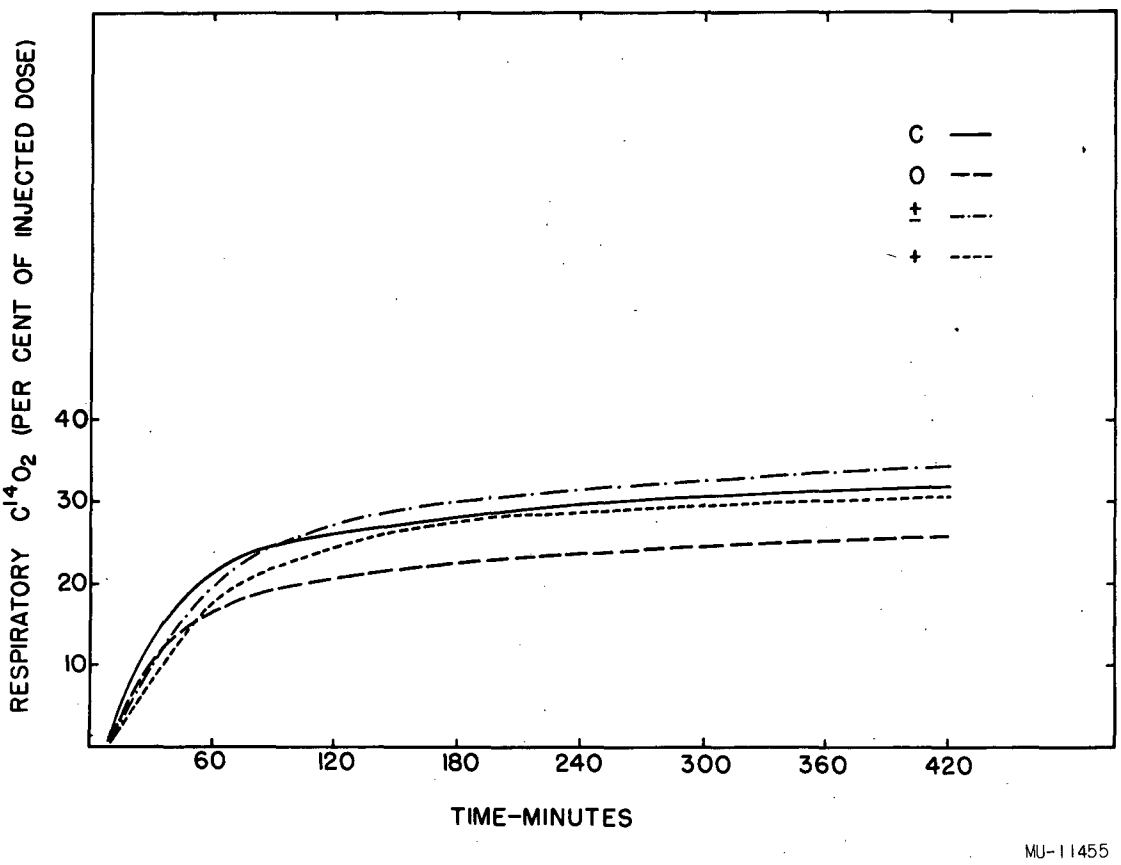

Fig. 8. Cumulative excretion of $C^{14}O_2$ in expired air over a period of 420 min following injection of L-methionine-methyl-C^{14}.

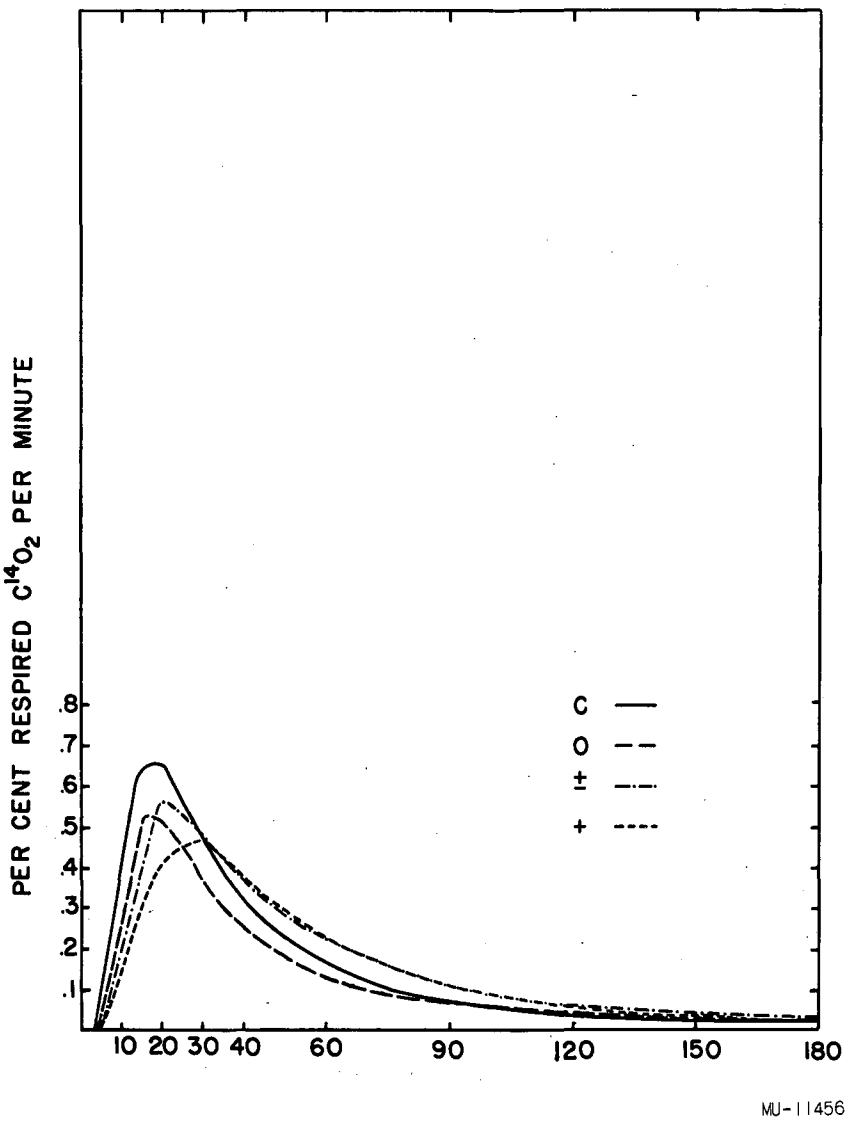

Fig. 9. Rate of excretion of $C^{14}O_2$ in expired air for a period of 180 min following injection of L-methionine-methyl-C^{14}.

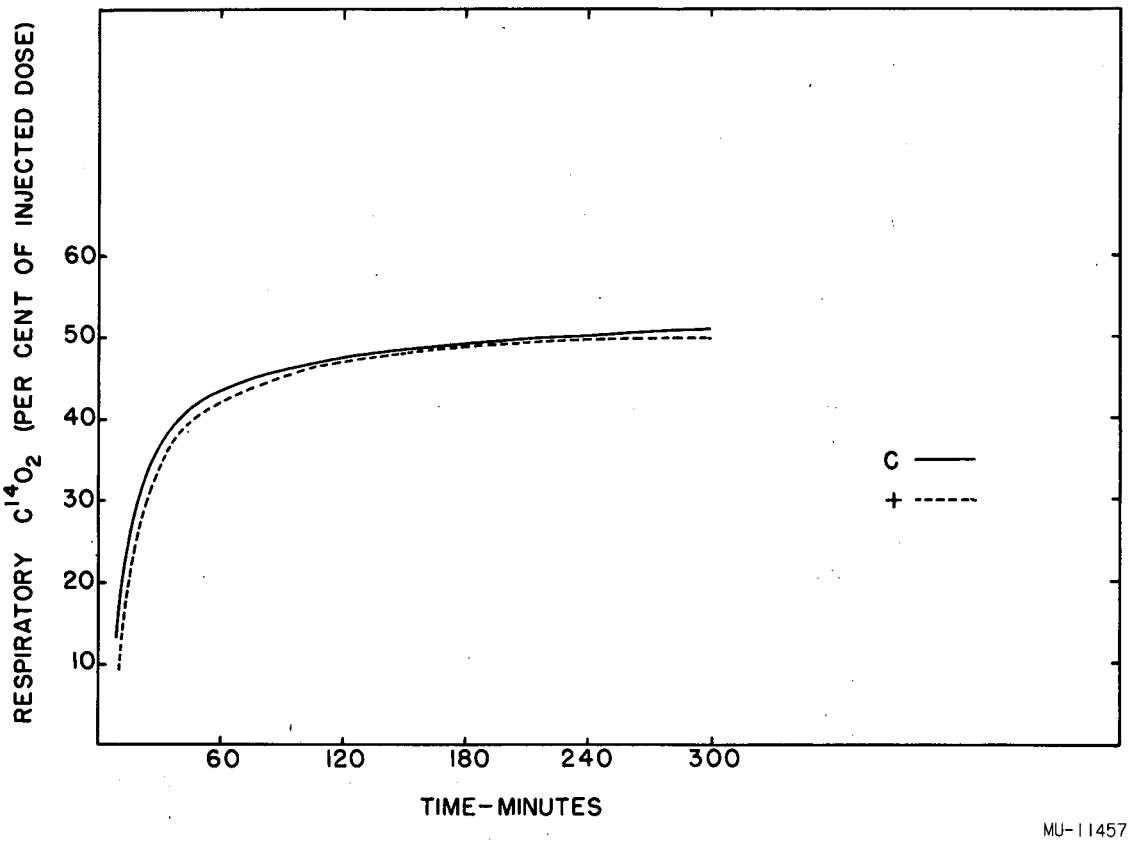

Fig. 10. Cumulative excretion of $C^{14}O_2$ in expired air over a period of 420 min following injection of DL-glutamic-1-C^{14} acid.

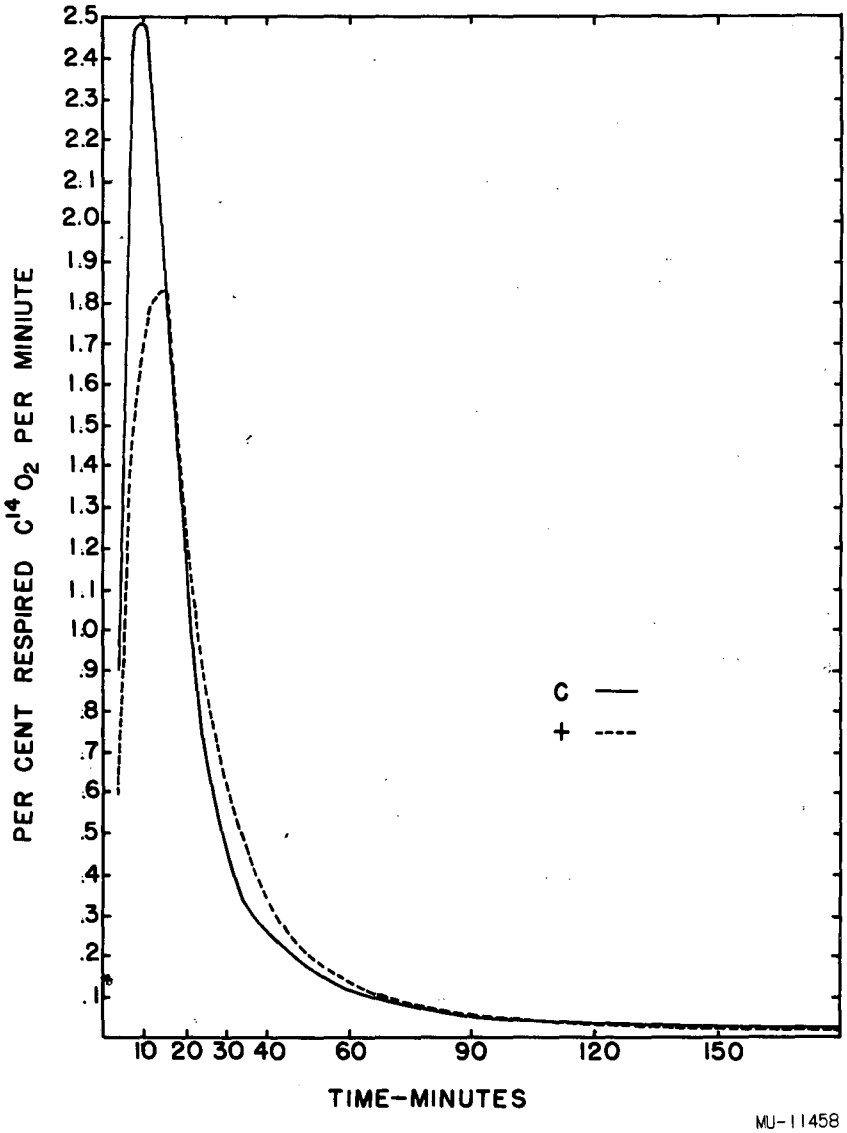

Fig. 11. Rate of excretion of $C^{14}O_2$ in expired air for a period of 180 min following injection of DL-glutamic-1-C^{14} acid.

the "D-L" mixture, a logical conclusion would be that the animal is capable of decarboxylating only one of the isomers, but at this time we are not sure which isomer is being rapidly decarboxylated.

These results together with previously reported data for glucose and acetate are most interesting, not because of the differences between the normal and sick animals, but because of the lack of any major differences. This would indicate that intermediary metabolism is not greatly changed in this disease state until at least a moribund condition is achieved. This generalized conclusion has also been observed in other illnesses, such as radiation sickness, mild starvation, and cancer.

A RAPID AUTOMATIC SAMPLER FOR USE IN METABOLIC STUDIES

Karl K. Lonberg-Holm

Previous investigation of the kinetics of glucose dissimilation in a free-cell neoplasm have indicated a need for rapid sampling (for analysis) of cell suspensions that have been fed glucose-C^{14} or given P^{32}. The changes occurring within 20 seconds of adding glucose to such a suspension are of interest.

To achieve such rapid sampling, an automatic device has been constructed that permits measured samples to be removed at few-second intervals and killed in hot ethanol. The "automatic rapid sampler" is built around a modified stopcock (see Figs. 12, 13, and 14). The plug of the stopcock (a) is Teflon and must be very carefully fitted to the glass shell. The final fit is improved by heating the stopcock to about 60° and turning, with pressure, the Teflon plug in the glass shell. The stopcock shell is modified from a 3-way (120°) Pyrex stopcock shell with about 1.5 cm inner diameter by the addition of an extra outlet (c) at 60° between e and d. Also a groove (s) is carefully placed at a little less than 60° from b and a little more than 60° from e (about the distance of the diameter h from the midway mark). The location of s (which is perpendicular to the plane of the paper in Fig. 12 and parallel in Fig. 13) is critical, and the dimensions of h, s, and d outlets may have to be adjusted empirically so that the operating cycle (to be described below) will be complete at the desired operating speed. Groove s must be so located that it is connected by h to d, but its position must also be such that when h is turned further in a clockwise direction d will connect only to h and s will be sealed off. Groove s connects with the air space g (Fig. 13).

The principle is as follows: The stopcock is driven by a motor and gear train or turned by hand at the desired speed. Outlet e is the reaction vessel with the cell suspension under controlled gas phase. Outlet c has water under pressure, d is a vacuum (aspirator), b leads to the preheated collection tubes with ethanol in them. As the hole h, which will contain water, meets d the water will be sucked out, because air can get in from g via s. As the rotation continues h becomes completely open to d but out of contact with s, and a vacuum is created. As h contacts e it fills its

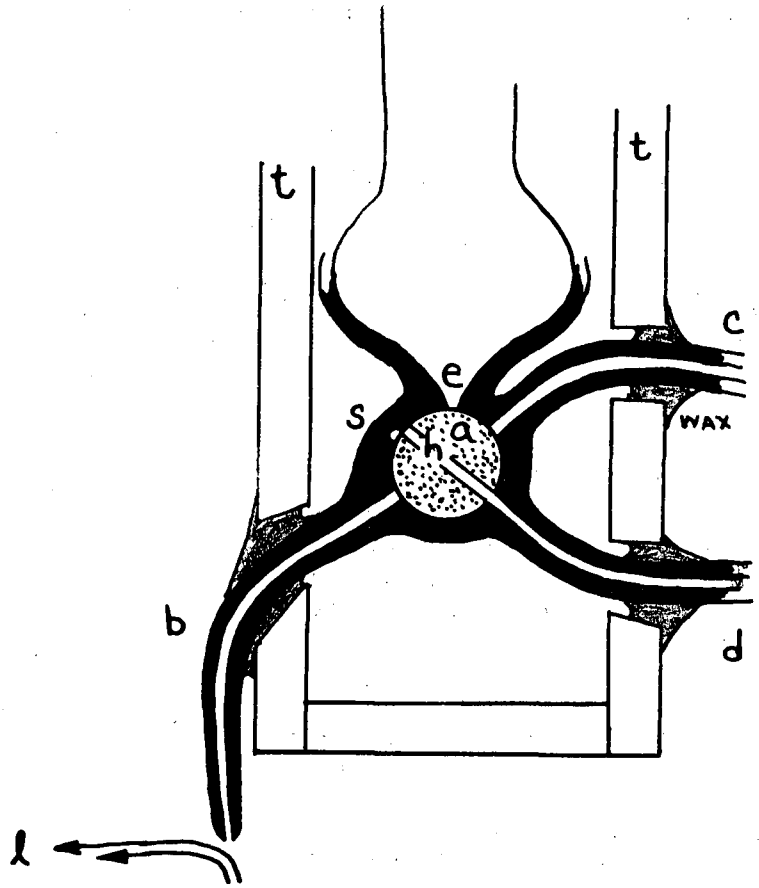

Fig. 12. Diagrammatic cross section of the automatic rapid sampler. The plug (a) is made of Teflon and the entire stopcock is in a lucite water jacket (t). Outlets b, c, and d are passed through oversized holes in t to avoid stress, and the holes are sealed with wax.

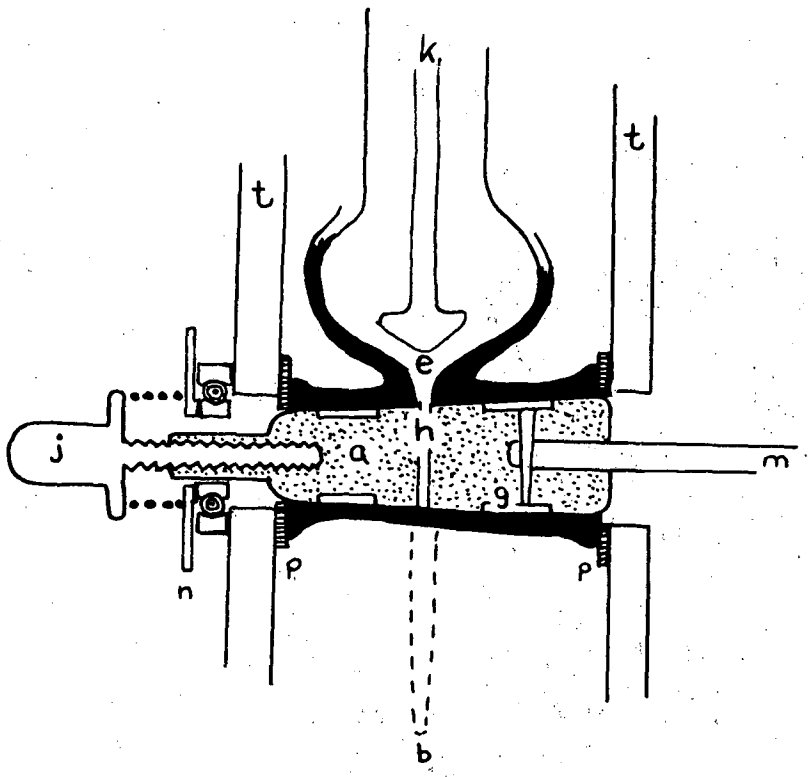

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Fig. 13. Diagrammatic cross section of the automatic rapid sampler (side view). The stopcock is turned by a gear on m. Drive shaft m is held in the Teflon plug by a tapered pin. The plug is held firmly in the sheath by a spring pressing against cam j and washer n. Washer n turns on a ball bearing resting directly on t. Support of the stopcock inside the jacket is provided by the faces being pressed against rubber gaskets (p) held against t. (The jacket is constructed about the stopcock and held together with glue and screws, the outlets shown in Fig. 12 being sealed with wax last of all in construction.)

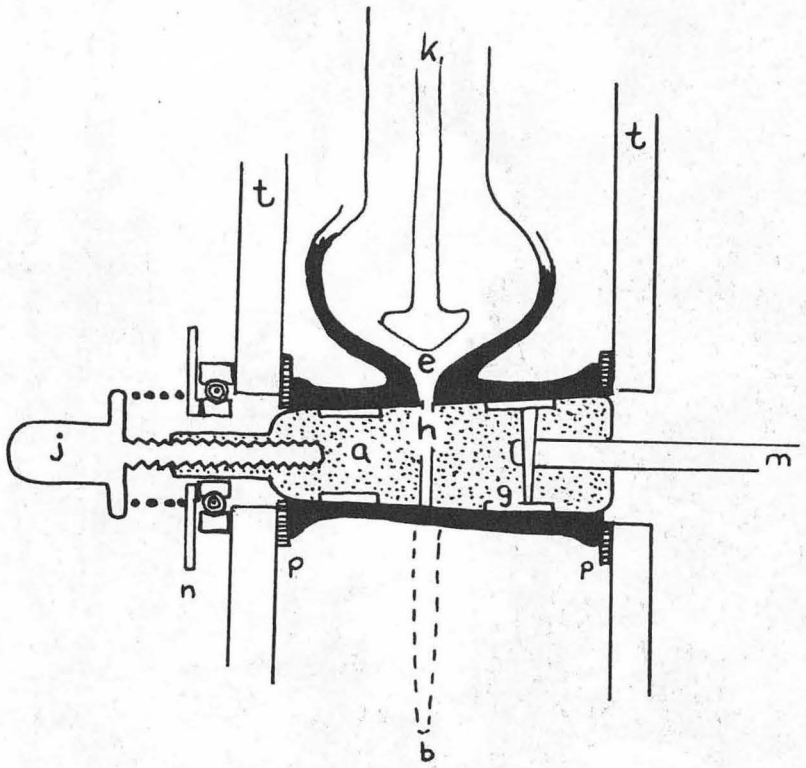

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Fig. 13. Diagrammatic cross section of the automatic rapid sampler (side view). The stopcock is turned by a gear on m. Drive shaft m is held in the Teflon plug by a tapered pin. The plug is held firmly in the sheath by a spring pressing against cam j and washer n. Washer n turns on a ball bearing resting directly on t. Support of the stopcock inside the jacket is provided by the faces being pressed against rubber gaskets (p) held against t. (The jacket is constructed about the stopcock and held together with glue and screws, the outlets shown in Fig. 12 being sealed with wax last of all in construction.)

volume with cells, which are then flushed out with water at c via b and into hot alcohol. Then a second cycle begins. There are two cycles per complete revolution. The volume of the sample is determined by the hole h. In our machine h is 25 microliters and the precision of delivery volume is about 1% at a frequency of 1.6 seconds per cycle.

The collection tubes are in a block of dural equipped with heating element, and can be pulled along in the block by a weight and pulley. The progression of the block is controlled by a solenoid-driven shuttle. The solenoid is activated by a microswitch riding a cam, j, on the stopcock plug, and is coordinated with the cycle by its angular location about j.

The entire stopcock is water-jacketed (t) for temperature control. It is also equipped with a stirring rod (k), which is turned at a slow speed by a stirring motor. As the sample is flushed out it makes contact between two platinum wires and provides a signal for an Esterline Angus recorder (l) traveling at top chart speed, thus keeping a record of sampling time.

Devices of this type may be of use in studying other moderately rapid systems in organic chemistry and biochemistry.

Mr. Edward Pauletch of the UCRL Machine Shop and Mr. Harry Powell of the UCRL Glass Shop gave their excellent skills toward the construction of this device, and I wish to thank them also for their many suggestions.

Calvin

TRACER DIFFUSION IN WATER

Elton M. Baker

Scintillation counting methods applied to the detection of tritium-labeled water in aqueous solutions are more convenient and rapid than the alternative method of generating hydrogen gas from the water and detecting its activity in an iron chamber. Furthermore, in studies where the exchange of tritium with hydrogen in other compounds is negligible, a scintillation procedure provides a convenient method of following changes in concentration of water in solutions.

Studies of tracer diffusion using tritium-labeled water have been started with modifications of the methods of Wang[1] and of Burkell and Spinks.[2] Briefly, the method of free diffusion used here utilizes small capillaries closed at one end and held horizontally submerged in a volume of water of not less than 2 liters. The water is not stirred, and diffusion takes place from the open ends of the capillary tubes into a nearly infinite volume, with almost no backward diffusion of the tritium-labeled water. The use of small capillaries has the special advantage that vibrations or convection currents in the large liquid volume do not cause errors in the measurements.

The tubes used are 4 cm in length and 0.75 mm in diameter. One end of each is fastened into a small lucite holder which provides for four separate tubes. Each tube is held in a small opening in the circular holder by means of a lucite collar. A small Teflon disc is held tightly against the lower end of the tube and sealed with silicone grease if needed.

The capillaries are filled with tritium-labeled water of known concentration, are placed in the lucite holder, and are lowered carefully into the large volume of water to within a millimeter of the open end. After the solution has come to constant temperature the entire holder is carefully submerged, by means of a small screw attached to the holder, so that turbulence will not be set up at the open ends of the capillaries. Diffusion from the capillaries is allowed to proceed until approximately 1/4 of the original activity remains. This requires 3 or 4 days for water at 25°C. The capillaries are then removed and their outer surfaces dried carefully. The liquid remaining in each capillary is removed with a small syringe and hypodermic needle which has had its tip cut off to give a smooth end. Each capillary is washed several times until it is free of activity. The water and washings are then diluted to a known volume and analyzed by means of a coincidence scintillation counter.[3]

The initial results for tracer diffusion of water are, under these conditions, by Fick's second law,[4]

$$\frac{\partial C}{\partial t} = D \frac{\partial^2 C}{\partial X^2} , \qquad (1)$$

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[1] J. H. Wang, J. Am. Chem. Soc. 73, 510 (1951).

[2] J. W. Burkell and J. Spinks, Can. J. Chem. 30, 311 (1952).

[3] E. M. Baker, in UCRL-3240, Jan. 1956, p. 39.

[4] A. Fick, Ann. Physik. 94, 59 (1855).

Calvin

where C is the concentration of molecules at time t, X is the coordinate along which diffusion takes place, and D is the diffusion coefficient, which is assumed to be independent of tracer concentrations, and leads to

$$\frac{C_{ave}}{C_0} = \frac{8}{\pi^2} \sum_{n=0}^{\infty} \frac{1}{(2n+1)^2} \exp\left[-\frac{(2n+1)^2\pi^2 Dt}{4\ell^2}\right] . \qquad (2)$$

where C_0 is the initial concentration of the tracer molecule, C_{ave} is the average concentration in the capillaries at time t, (and n is as used in the Fourier series). When

$$\frac{Dt}{\ell^2}$$

is large, the series on the right side of the equation converges rapidly and the terms after the first can be neglected with an error less than that of determining the activity by the scintillation counter. Then Eq. (2) may be written in a more usable form:

$$D = \frac{4\ell^2}{\pi^2 t} \ln\left[\frac{8}{\pi^2} \times \frac{C_0}{C_{ave}}\right] . \qquad (3)$$

Preliminary results gave 2.44 ± 0.18 cm^2/sec for the tracer diffusion of water. Studies will continue with tracer diffusion; other labeled compounds will be used in an attempt to learn more about the liquid structure of solvents. Some studies of water transport and diffusion are to be made in an electric field.

RADIATION DECOMPOSITION OF PORPHINS

Ann C. Kleerup and B. M. Tolbert

Most organic compounds have a G(-M) value for radiation decomposition around 5 to 10; that is, 100 ev of ionizing radiation energy will cause 5 to 10 molecules to be permanently altered in a chemical sense. In the preceding quarterly report of this group, an anomolous radiation stability for hemin was reported--the G(-M) value was found to be not greater than 0.17.[1] Since then, this work has been extended to other compounds containing the porphin ring and these compounds also are seen to exhibit this same unusual stability.

Copper phthalocyanine in the α-crystalline form was obtained in a very pure state. Anal. Calcd. for $C_{32}H_{16}N_8Cu$: C, 66.72%; H, 2.80%. Found: C, 66.86%; H, 2.77%. Weighed amounts of dry α-copper phthalocyanine were sealed in vacuo in 4-mm od Pyrex tubes. Two samples were not irradiated. The others were irradiated in the Co^{60} source. After the samples had been irradiated, the weighed tubes were opened, the absence of any appreciable gaseous decomposition products shown, and then a 0.5-mg aliquot of each sample was weighed out. The samples were each dissolved in a liter of 1-chloronaphthalene (solubility about 2.5×10^{-6} M/l) and analyzed spectrophotometrically. As shown in Table IX, the apparent decomposition was in each case within the experimental error of ± 1%.

Zinc tetraphenylporphyrin crystals were irradiated in the same way and dissolved in benzene for spectroanalysis. These results are less reliable because of possible photooxidation of the compound after being put into solution. The several spectra, although very similar, did not exhibit identical ratios of maxima to minima or of maxima to maxima. However, the extensive decomposition that would have occurred in a normal organic compound, G(-M) = 5, did not appear. For this reason the approximate amounts of decomposition are included in Table IX.

Nonmetallic phthalocyanine has been recrystallized from 1-chloronaphthalene and from quinoline. Samples are now being irradiated in the Co^{60} gamma source. Attempts to purify protoporphyrin have so far proved unsuccessful.

The determination of radiation decomposition G values for these porphins has thus far been hampered by three factors. We had no idea how much radiation energy would be required to produce a significant percent decomposition, we had very limited high-intensity radiation sources, and we were not familiar with the purification and analysis of these compounds. The last problem we have solved. We now have two radiation sources not previously available, namely, a 3-Mev electron linear accelerator capable of delivering 10^9 rep/hr, and the possibility of using the high-intensity gamma field in the spent fuel element tank at Arco. We are planning on irradiating these porphin samples with 10^9 to 10^{11} rep of ionizing radiation.

[1] Ann Kleerup and B. M. Tolbert, in UCRL-3351, March 1956, p. 4.

Calvin

Table IX

Irradiation decomposition of hemin by Co^{60} gamma rays

Compound	Amount of radiation (rep)	Decomposition (%)	Analytical Procedure
Copper phthalocyanine	1.2×10^7	0.5	Spectra in 1-chloronaphthalene
Copper phthalocyanine	1.2×10^7	1	Spectra in 1-chloronaphthalene
Copper phthalocyanine	1.3×10^8	1[a]	Spectra in 1-chloronaphthalene
Copper phthalocyanine	1.3×10^8	0.3	Spectra in 1-chloronaphthalene
Zinc tetraphenyl-porphyrin	1.3×10^7	< 1	Spectra in benzene
Zinc tetraphenyl-porphyrin	1.3×10^7	< 1	Spectra in benzene
Zinc tetraphenyl-porphyrin	9.6×10^7	< 1	Spectra in benzene
Zinc tetraphenyl-porphyrin	9.6×10^7	< 1[b]	Spectra in benzene

[a] This corresponds to a G(-M) value of less than 0.2.

[b] This corresponds to a G(-M) value equal to or less than 0.18.

OXIDATION OF SODIUM SALTS OF ORGANIC ACIDS

Irville M. Whittemore and Bert M. Tolbert

Wilzbach and Sykes[1] have described a sealed-tube combustion techniques for converting organic compounds to CO_2, using CuO as the oxidizing agent with a little added copper metal as a scavenger for oxygen and oxides of nitrogen. We have found, as they did, that this method is quantitative for many classes of compounds. However, when a sodium salt such as sodium acetate is oxidized in this way generation of the CO_2 is not complete, and it is thought that the following reaction may be taking place:

$$8\ CuO + 2CH_3CO_2Na \rightarrow 8\ Cu + H_2O + 3\ CO_2 + Na_2CO_3. \qquad (1)$$

There is no acid present to react with the sodium carbonate, and as much as 25% of the CO_2 is not recovered for analysis. If SO_3 could be added to react with the sodium carbonate a quantitative yield might be obtained; sufficient SO_3 to react with the copper metal would have to be added. If the extra scavenger copper were left out then we would have

$$9\ SO_3 + 8\ Cu + Na_2CO_3 \rightarrow 8\ SO_2 + 8\ CuO + Na_2SO_4 + CO_2. \qquad (2)$$

As $CuSO_4$ decomposes into $CuO + SO_3$ at the temperature of the combustion (640°C), it was decided to use anhydrous $CuSO_4$ as the source of SO_3:

$$CuSO_4 \xrightarrow{650°C} CuO + SO_3. \qquad (3)$$

The over-all total of Eqs. (1), (2), and (3) is simply

$$9\ CuSO_4 + 2\ NaOOCCH_3 \xrightarrow{650°} 4\ CO_2 + 3\ H_2O + 8\ SO_2 + Na_2SO_4. \qquad (4)$$

A combustion using only $CuSO_4$ in 50% excess as an oxidant was therefore tried and found to yield CO_2 quantitatively within experimental limits (based on $C^{14}O_2$ yield from 1-labeled acetate).

Thus far only sodium acetate has been burned by this method. It is planned to investigate other alkali salts.

Measurements of $C^{14}O_2$ were made in ionization chambers with CO_2 as the diluting gas. The SO_2 formed in Reaction (2) also passed into the chambers under the conditions used, and forms about 1%-2% of the total gas content of the chamber. What effect the SO_2 has on the ion-pair yield in the chamber has not yet been determined, but it is presumed to be small.

It is to be noted that in this method a considerable amount of gas is formed in the combustion. In our work, a 10-cc sealed tube of Pyrex 1720 glass was calculated to contain 3 to 5 atmospheres of gas at 650°C. Great care must be taken to construct tubes of sufficient wall thickness to withstand this pressure. As a precaution, the sealed tubes should be placed inside a closed metal tube during the reaction in the furnace. The glass tube should, of course, be evacuated before sealing, and anhydrous $CuSO_4$ used as to minimize the pressure.

[1] K. E. Wilzbach and W. Y. Sykes, Science 120, 494-496 (1954).

EFFECTS OF IONIZING RADIATION ON CHOLINE ANALOGS

Richard M. Lemmon, Peggy Kwong, Franco Mazzetti, and Margaret A. Parsons

In a previous quarterly report[1] the considerable radiation stability of choline acetate was reported. This compound was of particular interest because of the proton affinity of the acetate ion. As an extension of this work we have prepared and irradiated choline cyanide,

$$\left[(CH_3)_3NCH_2CH_2OH\right]^+CN^-.$$

The cyanide ion has a greater proton affinity than does acetate; in addition, it is a considerably smaller ion, smaller even than the chloride. These two properties led to the supposition that choline cyanide might be more radiation-sensitive than choline chloride.

The choline cyanide was prepared by passing a solution of choline hydroxide through a Dowex-2 anion-exchange column; the column had been previously converted to the cyanide form with aqueous hydrocyanic acid. The effluent was evaporated to dryness and the residue was recrystallized from ethanol-ether, giving analytically pure choline cyanide. Samples of the anhydrous, crystalline compound were subjected, under high vacuum, to the electron beam of a linear electron accelerator. Other samples were irradiated with the γ-radiation of a cobalt-60 source. The conditions under which the irradiations were performed and the analytical procedures (reineckate analysis) used to determine the extent of radiation decomposition were described in an earlier report.[2] At the present time three electron irradiations have given G values (molecules decomposed per 100 ev) of 15, 19, and 23. One gamma-ray irradiation has been performed giving a G value of 16. It is therefore appears that choline cyanide's radiation sensitivity is only approximately one-tenth that of choline chloride; that is, the radiation stability is greatly enhanced by replacing the chloride with a smaller anion. Increased stability is also apparent with larger anions (i.e., NO_3^-, I^-, $SO_4^=$). These data further emphasize that the radiation sensitivity of choline chloride must be a function of some unusual aspect (as yet unknown) of its crystal structure.

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1 Richard M. Lemmon, Peggy Kwong, and Margaret A. Parsons, UCRL-3240, Dec. 1955, p. 21.

2 Richard M. Lemmon and Margaret A. Parsons, UCRL-2647, July 1954, p. 4.

Calvin

SYNTHESIS OF LABELED COMPOUNDS

A. Preparation of $HC^{14}N$ from $BaC^{14}O_3$

Peggy Kwong and Richard M. Lemmon

By the procedure of Sixma et al.,[1] 2.8 mmoles of C^{14} labeled sodium cyanide was prepared as follows: Six 91-mg portions of $BaC^{14}O_3$ (71 μc/mg) were weighed into each of six separate tubes (15 by 1.1 cm od) of Pyrex 1720 (high-temperature) glass. Fifty milligrams of ammonium chloride was added to each tube and the air was partially displaced with a slow stream of nitrogen. Approximately 1 gram of metallic potassium (freshly cut under ether) was added to each tube, and the tubes were alternately evacuated and filled with nitrogen several times. The tubes were then sealed off and placed in an oven at 640° for 75 minutes. After the tubes were cooled they were broken open separately and the excess potassium decomposed with absolute ethanol. Excess water was added and the solution made acid with sulfuric acid. The solution was then boiled until about 15 cc of water was distilled; the outlet tube dipped below a 1 N NaOH solution. The yields were determined by titrating an aliquot portion of the alkaline solution after first adding excess NH_4OH and one KI crystal. In the six preparations the cyanide yields varied from 90% to 110%. The above-theoretical yield is due to absorption of CO_2 (from the air) in the original reaction tube. The specific activity of the cyanide was not determined.

B. Synthesis of Sodium Decanoate-1-C^{14}

Masao Nakazaki and Patricia T. Adams

Sodium decanoate-1-C^{14} ($n\text{-}C_9H_{19}C^{14}OONa$) has been prepared for use in biological experiments to be described later. Examination of the literature indicated that this compound had not been previously prepared. The carboxyl-labeled acid was obtained by the usual carboxylation[2] of n-nonyl magnesium bromide with carbon-14 dioxide. The formation of the emulsion that always is experienced in the extraction of higher fatty acids from organic solvent was easily suppressed by using a rather concentrated sodium hydroxide solution.

n-Nonyl bromide: A mixture of 200 ml benzene and 27.6 g phosphorus tribromide was added dropwise during 2 hr to a stirred solution of 21.81 g n-nonyl alcohol in 60 ml of dry benzene. The reaction temperature was kept at 60°-70°, and stirring at this temperature was continued another 2 hr.

The reaction mixture was poured onto 300 g of crushed ice and extracted with benzene (200 ml). After washing with water, sodium bicarbonate solution, and water, the solution was dried over calcium chloride. Bp, 91°-93°/7 mm Hg (bath temp. 110°), yield: 31.3 g (70%) n_D^{22} 1.4525.

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[1] F. L. J. Sixma et al., Rec. Trav. Chim. 73, 161 (1954).

[2] W. G. Dauben, J. Am. Chem. Soc. 70, 1376 (1948).

Carboxylation; n-Decanoic acid, low specific activity. The Grignard solution was prepared with 2.155 g of n-nonyl bromide and 0.251 g of magnesium in 30 ml of absolute ether. This Grignard solution was diluted with ether to 100 ml. An aliquot 2 ml was titrated with 0.1 N hydrochloric acid with methyl orange as indicator.[3]

This Grignard solution (98 ml, 0.0875 N) was carboxylated at -20°C by the radioactive carbon dioxide that was generated from 1.069 g of barium carbonate (5.42 millimole) containing 84 μc of radioactive barium carbonate. The decanoic acid was extracted from the reaction mixture according to the usual procedure (using 1 N sodium hydroxide solution for extraction of the acid). The isolated acid was titrated with 0.1 N sodium hydroxide by potentiometric titration and lyophilized. Yield: 4.633 millimole (86%). Specific activity: 0.07 μc/mg (calcd, 0.086 μc/mg).

n-Decanoic acid, high specific activity: The Grignard solution (98 cc; 0.088N; prepared from 2.162 g of bromide and 0.253 g of magnesium) was carboxylated by the carbon dioxide generated from 0.1709 g of barium carbonate (5.95 millimole, 3.75 mc).

The yield of n-decanoic acid was 3.33 millimole (56%). Specific activity: 3.0 μc/mg (calcd, 3.4 μc/mg).

Paper chromatographic analysis (using n-butanol saturated with 0.5 N aqueous ammonia as solvent) showed a single spot, R_f 0.81. No other radioactive spots were detected on the radioautograph.

C. Thioctic-S_2^{35} Acid and Derivatives

Masao Nakazaki

Sulfur-labeled thioctic acid was prepared according to the method of Patricia T. Adams,[4] through the following sequence of compounds:

Benzyl mercaptan-S^{35}: Benzyl magnesium chloride (12 millimole in 25 ml ether) was added to the benzene suspension (18 ml) of 160 mg (5 millimole) sulfur containing 50 mc of S^{35} (Oak Ridge National Laboratory). Yield: 74%.

6,8-Dibenzylmercapto-S_2^{35}-octanoic acid: yield, 69%; specific activity, 38.4 μc/mg (calcd, 51.5 μc/mg).

6-Thioctic-S_2^{35} acid: Yield, 29.4%; mp., 60-61°. Specific activity, 50 μc/mg (calcd, 72.3 μc/mg).

In order to study the thioctic acid lipid which was found as a metabolite of thioctic acid in algae, it seemed desirable to prepare some esters of thioctic acid as model compounds. But, because of sensitivity to the action of acid and heat, esters of thioctic acid have not been prepared by usual esterification methods, and some attemps were made in vain. The methyl

[3] H. Gilman et al., J. Am. Chem. Soc. 45, 150 (1923).

[4] Patricia T. Adams, J. Am. Chem. Soc. 77, 5357 (1955).

ester of thioctic acid was prepared by methylation with diazomethane,[5] but this method would be very difficult for the preparation of esters of higher alcohols, especially phytol.[6]

As a relatively mild esterification method, Freudenberg's procedure[7] was tried. This consisted of treatment of thioctic acid in methanol with acetyl bromide at room temperature. Methyl thioctate was obtained in good yield. This method may be useful for preparation of some esters or glycerides of thioctic acid for the further study of the nature of thioctic acid lipid.

Methyl thioctate: To the solution of thioctic acid (60 mg) and 4 ml of methanol, 20 mg of acetyl bromide was added, and the reaction mixture was kept at room temperature (25°) overnight. A slight turbidity (thioctic acid polymer?) became apparent during this period. The solution was poured on crushed ice (20 g) and extracted with benzene, the benzene layer was washed with water, diluted sodium bicarbonate solution, and water successively, and was dried over magnesium sulfate. The yellow viscous liquid that remained after evaporation of the solvent was purified by vacuum distillation (0.01 mm Hg, bath temperature 70° C) in a sublimation apparatus, giving 50 mg yellow liquid (78%) ($n_D^{23°}$ 1.5248).

Infrared spectra: 1750 cm^{-1} (CO); 2950 and 2830 cm^{-1} (CH_3); O-H band, none.

This product was subjected to paper chromatography in n-butanol saturated with 0.5 N aq. ammonia, after which the paper was sprayed by sulfur spray solution. Almost all the sulfur compound was found at the solvent front, with a small amount of sulfur compound detected on the origin (polymer of the thioctic acid).

Methyl thioctate eluted from the paper chromatogram with methanol was hydrolyzed by heating on a steam bath with 3 N hydrochloric acid. After 2 hours the solution was extracted with ether, and the extract was chromatographed on paper with n-butanol saturated with 0.5 N aq. ammonia as solvent. Almost all the sulfur compound was found at R_f 0.42 (thioctic acid sulfoxide) except for very small amounts of thioctic acid at R_f 0.76.

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5 Gunsalus, Burton, and Gruber, J. Am. Chem. Soc. 78, 1763 (1956).

6 On the instability of vinyl diazomethane, see C. D. Hurd and S. C. Lui, J. Am. Chem. Soc. 57, 2656 (1935).

7 K. Freudenberg and W. Jakob, Ber. deut. chem. Ges. 74, 1001 (1941).

D. The Resolution of C^{14}-Labeled Amino Acids

Patricia T. Adams

In a previous quarterly report[8] a method for the resolution of DL-valine was described. This procedure (consisting of the enzymatic hydrolysis of the L-chloroacetyl amino acid) has been employed to resolve high-specific-activity samples of leucine, norleucine, and norvaline.

A simplified method of isolating the pure optical isomers from the reaction mixture through the use of Dowex-50 ion-exchange resin has been developed. The crude reaction mixture after enzymatic hydrolysis of the L-acyl amino acid contains about 1 g of total amino acid and 3 to 5 g of sodium chloride. This mixture is run through a column containing about 100 cc of Dowex-50 resin in the hydrogen ion form. Acyl-D-amino acid and chloride ions are washed from the column with 300 ml water. The L-amino acid, completely free from sodium ion contamination, is eluted from the column with 200 ml of 1 M ammonium hydroxide. Filtration of the eluate and evaporation to dryness give directly the pure salt-free L-amino acid. (The resin is prepared for subsequent re-use by elution of sodium from the column with hydrochloric acid.)

The D-isomer is obtained by hydrolysis of the acyl D-amino acid in 6 N hydrochloric acid. The hydrolysis mixture is again run through the resin column. Chloride is removed in the water effluate and pure D-amino acid is isolated from the ammonium hydroxide eluate.

By this procedure, the following amino acids have been prepared:

From DL-leucine-3-C^{14} (636 mg; 3.4 μc/mg) there was obtained 177 mg of D-leucine-3-C^{14} (3.4 μc/mg; $[\alpha_D]^9 = -15.6$) and 193 mg of L-leucine-3-C^{14} (3.4 μc/mg; $[\alpha_D] = +15.8$) for a 58% recovery.

From DL-norleucine-3-C^{14} (790 mg; 4.2 μc/mg) there was obtained 263 mg of D-norleucine-3-C^{14} (4.2 μc/mg; $[\alpha_D] = -21.8$) and 290 mg L-norleucine-3-C^{14} (4.1 μc/mg; $[\alpha_D] = +22.8$) for a 70% recovery.

From DL-norvaline-3-C^{14} (720 mg; 4.9 μc/mg) there was obtained 225 mg of D-norvaline-3-C^{14} (4.9 μc/mg; $[\alpha_D] = 23.4$) and 283 mg L-norvaline-3-C^{14} (4.9 μc/mg; $[\alpha_D] = +22.8$) for a 70% recovery.

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[8] Kirk, Adams, and Tolbert, in UCRL-3351, March 1956 p. 21.

[9] All optical rotations measured in 6 N HCl.

NUCLEAR CHEMISTRY

Glenn T. Seaborg and Isadore Perlman in charge

MASS SPECTROSCOPY

Fred L. Reynolds and Maynard C. Michel

Eight-Inch Spectrometer

During the last quarter, work has been completed on the conversion of the 8-inch-radius 60° mass spectrometer to a relatively high-sensitivity abundance-measurement machine. This was originally built in 1948 as an oil-pumped, rubber-gasketed instrument for use with both photographic and ion-current detection and was later modified for low-gain electron multiplier detection. Its usefulness for high-sensitivity work (samples of 10^{-8} gram or less) was severely limited by a pressure of 10^{-6} mm and organic contaminants, so that a rather extensive revision was planned, to be concurrent with the construction of the 12-inch-radius instrument now nearing completion.

Modifications were primarily in the vacuum chamber. All parts were made of stainless steel and were copper-gasketed; mercury pumps have been substituted for the oil pumps, and a system of remotely soldered indium valves provided to allow sample insertion with a minimum amount of the system exposed to air. (See Quarterly Report, UCRL-3351) In addition, a high-gain (5×10^9 max) multiplier (Ag-Mg) has been added to bring single-ion detection well within the limits of the instrument. Slight modifications to the electronics were made and a proton resonance fluxmeter added as in our other instruments.

Present operation is quite satisfactory in most respects; however, some changes in ion-source geometry are in order. The detection equipment has a background counting rate of about 30 cpm. With a tungsten filament at 3000° K, the usual background peaks from K polymers, alkali impurities and W^+ ions are seen; but in the clear regions, the counting rate approaches the background rate after initial cleanup of the source.

With this sensitivity level and with multiple-filament thermal ion sources, we have been able to utilize with ease samples of the heavy elements averaging 10^{-9} gram. The precision of ratios is considerably affected, however, by the relative abundance within the sample, since beam currents of 10^{-17} ampere or less, with their accompanying statistical fluctuation, are utilized for most low-abundance isotopes. In mixed crystals, it has been possible to see heavy (transuranium) elements present in total mass of less than 10^{-12} gram, although measurements of abundance suffer from this statistical fluctuations of the ion beam.

Use of slow-electron bombardment sources results in considerable organic background current, partly unavoidable because of the necessity of letting down to air for admission of each sample. The level of this background is of the order of 10^{-17} to 10^{-18} ampere for the mass region above 150, with a peak appearing at essentially every mass number. No attempt

has been made to determine the disappearance of these with continued outgassing of the walls, although previous experience would lead us to believe that much of this background is still due to incomplete initial outgassing of the machine; a lower level may be possible upon longer pumping.

Principal use of the 8-inch mass spectrometer will be in mass analyses of highly active samples (to relieve the 12-inch machines from contamination) and in a number of high-temperature liquid-gas equilibrium studies on transuranium metals, for which purpose quartz windows are being installed in the source so as to permit temperature measurement.

Time-of-Flight Spectrometer

The time-of-flight separator was used to separate and assign or confirm previous assignments of, Er^{165}, Er^{171}, Tm^{171}, and the new isotope Tm^{172}. All separations were successful and yields were consistent with previous experiments with these elements. No important discrepancies were noted in half lives or in assignments previously made, although data for the 1.9-yr Er^{171} are not complete. A more complete summary of this work appears elsewhere in this report as well as in UCRL-3286.

MS VI

The 12-inch-radius high-sensitivity instrument is approaching assembly and should be operating by late summer or early autumn. Its properties will be reported in the appropriate quarterly report.

A WINDOWLESS PROPORTIONAL COUNTER FOR THE MEASUREMENT OF LOW-ENERGY X-RAYS AND ELECTRONS

Marvin I. Kalkstein

Some work has been done on setting up and testing a windowless proportional counter. The counter used has an active volume about 16 inches long by 3 inches in diameter. Samples are introduced into the counting volume by means of a sliding sample holder. The counter is operated as a flow counter with a mixture of 90% argon and 10% methane as the counting gas. High voltage to the center wire is supplied by several 300-volt batteries. Usually eight or nine batteries are used. The signals are passed through a preamplifier to the 50-channel pulse-height analyzer.

Samples of Mn^{54}, Fe^{55}, Am^{241}, and Tc^{99m} have been used to help check the counter. The follwing results were obtained:

Sample	Radiation	Energy	Resolutions	Efficiency(assuming a geometry of 0.5)
Mn^{54}	Cr K x-rays	5.4 kev		> 0.25
Fe^{55}	Mn K x-rays	5.9 kev	~19%	
Am^{241}	$L\alpha_1$ x-rays	14 kev		~ ~ 0.2
	$L\beta_1$ x-rays	18 kev		~ ~ 0.1
	$L\gamma$ x-ray	21 kev		~ ~ 0.04
	60 kev γ-ray	60 kev		~ ~ 0.001
Tc^{99m}	electrons	< 2 kev		> 0.1

The efficiency for the Cr K x-rays is given a lower limit, since a "thick" precipitated sample was used. The result for the Tc^{99m} is also a lower limit because, although the electroplated sample appeared to be essentially weightless, the spectrum obtained was a continuum with a maximum energy of about 2 kev. As the range for electrons of this energy is of the order of a few micrograms, there is undoubtedly considerable absorption. In addition, there is some bias in the amplifier of the 50-channel pulse-height analyzer so that the very low-energy electrons are not counted.

THE NEW ELECTRON-CAPTURE ISOTOPE RHENIUM-181

Charles J. Gallagher, Jr., Frederick L. Canavan, Donald Strominger, and John O. Rasmussen

A series of experiments has led to the mass assignment of the approximately 22-hour electron-capture isotope of Rhenium as Re^{181}.[1]

The isotope was produced by two methods: deuterons on naturally occurring tungsten, and an (a, 4n) reaction on the naturally occurring Ta isotope Ta^{181}. To define the mass number, three bombardments were carried out with alpha particles on Ta^{181}: one at 24 Mev, one at 48 Mev, and a stacked-foil excitation function. The isotope was produced at 48 Mev and not at 24 Mev; the stacked foil indicated a threshold at about 38 Mev rather than the 32-Mev threshold calculated from the semiempirical masses by N. Metropolis and G. Reitwiesner.[2]

The half life of the isotope was determined by following the decay of the prominent 365.4 ± 0.3-kev gamma ray of the isotope on a single-channel gamma analyzer and also by observing the decay of the conversion electron lines of the same gamma in a double-focusing beta-ray spectrometer.

[1] Michael Sweeney and John Rasmussen, in Chemistry Division Quarterly Report, UCRL-2932, March 1955.

[2] N. Metropolis and G. Reitwiesner, Table of Atomic Masses, NP-1980 (1950), Technical Information Service, Oak Ridge, Tennessee, (1950).

The decay scheme of the isotope is not yet determined, owing to the extreme complexity of the gamma radiations of Re^{182} and Re^{183}. However, we are unambiguously able to assign the above-mentioned 365.4 ± 0.3-kev gamma to Re^{181}. Because of its prominence we postulate that all transitions occur through this state. Coincidence studies have indicated that the state in W^{181} giving rise to the 365.4-kev gamma has a half life longer than 0.1 microsecond.

The assignment of this line and the half-life measurement are in agreement with the recent betatron excitation studies[3] on natural tungsten performed at Iowa State College. In this work a 366-kev gamma ray was observed with a half life of 142×10^{-6} sec. We feel that we can unambiguously assign this gamma to a state in W^{181}.

[3] Stewart, Bureau, and Hammer, Bull. Am. Phys. Soc. 1, 206, R2 (1956).

Seaborg, Perlman

THE REACTIONS OF LANTHANUM-CERIUM TRIFLUORIDES WITH FLUORINE GAS

William P. Bryan

A study has been made of the reactions of mixed crystals of LaF_2 and CeF_3 with fluorine gas. Three independent methods were used to determine the percentages of Ce oxidized to the + 4 state when the mixed crystals were exposed to F_2 at 100°–300°C.

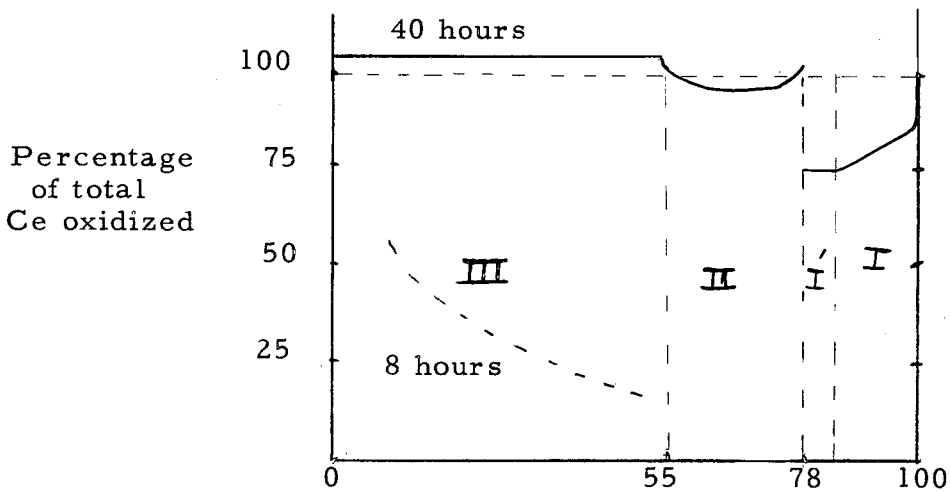
1. The change in wieght of the original $La_\delta Ce_{1-\delta}F_3$ sample upon exposure to fluorine gas was measured by weighing the sample in the nickel reaction pan before and after the reaction.

2. The nickel pan was suspended from a nickel helix in the fluorine atmosphere, and the deflections of the helix were measured during the course of the reaction. Knowing the helix sensitivity, one could calculate the weight increase of the sample.

3. The fluorinated materials were heated in air to convert them to oxides. Assuming

$$LaF_3 \rightarrow La_2O_3 \text{ and } \left.\begin{matrix} CeF_4 \\ CeF_3 \end{matrix}\right\} \rightarrow CeO_2,$$

one could calculate the amount of Ce in the +3 and +4 states. Results are summarized in the graph.



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Three separate regions were observed:

Region 1

X-ray pictures of the fluorinated materials showed one final phase: the monoclinic CeF_4 structure. The reaction was fast, approximately 10 minutes at temperatures between 100° and 200°. The reaction that occurs is

$$La_\delta Ce_{1-\delta} F_3 + \frac{1-\delta-\alpha}{2} F_2 = \underset{(CeF_4 \text{ structure})}{Ce^{IV}_{1-\delta-\alpha} Ce^{III}_{\alpha} La_\delta F_{4-\delta-\alpha}}. \quad (1)$$

In Region I' there may be a trace of some substance with the LaF_3 structure present in the final product, i.e.,

$$La_\delta Ce_{1-\delta} F_3 + \frac{1-\delta-\alpha}{2} F_2 = \underset{(CeF_4 \text{ structure})}{Ce^{IV}_{1-\delta-\alpha} Ce^{III}_{\alpha} La_{\delta-\beta} F_{4-\delta-3\beta-\alpha}} + \beta\, LaF_3 \quad (2)$$

Note the curious effect that as little as 0.2 atom % La causes α to go from 0 to approximately 0.1. After this about one Ce(III) is introduced for every one La(III) in the lattice.

Region 2

X-ray results show two final phases with the LaF_3 and CeF_4 structures. The reaction seems to go at about the same speed as in Region 1. A reaction that might occur is

$$La_\delta Ce_{1-\delta} F_3 + \frac{1-\delta}{2} F_2 = \delta\, LaF_3 + (1-\delta)\, CeF_4. \quad (3)$$

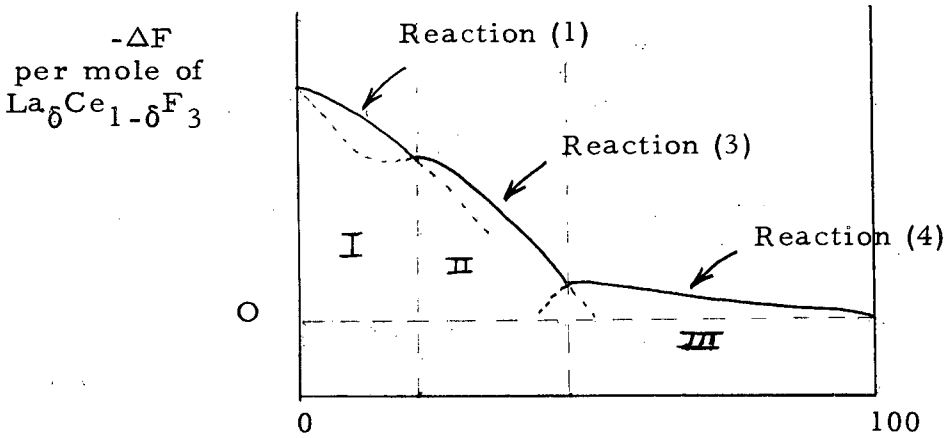
Region 3

Here the reaction is slow and takes about 40 hours to go to completion at 250°. X-ray results show only one final phase with the LaF_3 structure. Partially oxidized materials can be isolated upon shorter exposure to F_2. The complete reaction is

$$La_\delta Ce_{1-\delta} F_3 + \frac{1-\delta}{2} F_2 = \underset{(LaF_3 \text{ structure})}{La_\delta Ce^{IV}_{1-\delta} F_{4-\delta}}. \quad (4)$$

At present accurate unit cell dimensions for the various materials produced are being measured by the use of x-ray photographs and also by the use of a rotating Geiger tube and potentiometer which records the x-ray diffraction lines. Very accurate values of θ can be obtained by this method.

These results are unusual in that discontinuities exist in the curve for % Ce oxidized. On a thermodynamic basis such results might be explained by a situation where the free-energy curves for the various reactions cross. The diagram illustrates this idea.



In the various regions the solid lines represents the reaction that has the highest value of -ΔF.

THE CRYSTAL STRUCTURE OF AMERICIUM METAL

Peter Graf, Burris Cunningham, Carol Dauben,
David Templeton and Helena Ruben

A 5.73-cm-radius von der Hayde high-temperature x-ray camera has been put into operation. The temperature is constant within 5°C as measured by a thermocouple in place of the sample. This thermocouple reads 6°–10°C higher than the sample temperature determined by observing the transitions of $KClO_4$, Na_2CrO_4, and K_2CrO_4. The deviation is linear with temperature.

The americium metal used in these experiments was prepared by reducing AmF_3 with Ba in an all-Ta crucible system heated to about 1300°C in high vacuum. The product metal was annealed at 800°C for 4 hours and then cooled to room temperature at a rate of 1.50 to 2°C/min. The purity of the metal produced by this technique is more than 99.8%. Both Am^{241} and Am^{243} were used; the latter cut down the darkening of the film due to the sample radiations.

Four regions with different x-ray patterns are observed as the temperature is changed from 25°C to 800°C.

Region 1.

25°C to 75°–125°C: double hexagonal close packed, a=3.642 ± 0.005Å, c = 11.76 ± 0.01Å; alpha form of Am metal.[1]

Region 2

75°–125°C to 400°C: very poor diagram of a somewhat complicated, poorly crystallized form; this does not change nor improve with 12-hour heating at 350°C.

Region 3

400°C to 650°C: face-centered cubic, a=5.10 Å; gamma form of Am metal.

Region 4

650°C to 800°C: very complex pattern, which does not disappear on cooling.

Work is being continued in an attempt to identify clearly the phases on Regions 2 and 4.

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[1] Chemistry Division Quarterly Report for September, October, and November 1955, UCRL-3240.

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OPTICAL SPECTROSCOPY

John G. Conway, Ralph D. McLaughlin,
and George Shalimoff

Color centers in Crystals of Lanthanum Chloride Containing Ytterbium Chloride

Work has been started on color centers in $LaCl_3$ to which divalent Yb has been added. In the attempts to reduce the Yb from the $^{+3}$ state we have noted that it does not go as simply as did the Eu^{+3} to Eu^{+2}.[1] When 1% Yb is mixed into the $LaCl_3$, reduction does not take place even with prolonged heating in the presence of H_2, nor does the chloride thermally decompose on sublimation. If the Yb is physically separated from the $LaCl_3$ the reduction goes easily in a H_2 atmosphere at 650°C. Following the reduction, the $LaCl_3$ and $YbCl_2$ are sublimed together. The resulting mass turns blue-black when irradiated with ultraviolet light; it also phosphoresces for hours after the irradiation. Work is now under way to prepare a good single crystal so that measurements of the absorption spectrum may be obtained.

Hyperfine Studies

Procedures for the preparation of an Eu electrodeless discharge tube have been perfected to a point where it is possible to maintain a discharge with as little as 0.3 mg of Eu. Mass analysis of the Eu sample available indicates that the natural Eu^{153} is present in such high percentage (approx. 80%) that nothing can be learned of the nuclear properties of Eu^{154} and Eu^{155} by optical methods. The techniques developed will be applied to the investigation of other actinides and lanthanides.

[1] Gruen, Conway, and McLaughlin, Color Centers and Luminescence in Single Crystals of $LaCl_3$ Containing Eu^{+2}, UCRL-3249, Jan. 1956.

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SPALLATION OF THORIUM-232 WITH 20-TO 50-Mev HELIUM IONS

Bruce M. Foreman, Jr.

Thorium-232 is bombarded with helium ions from the Crocker 60-inch cyclotron in order to study the fission and spallation product yields and later the reaction mechanisms. Preliminary spallation cross sections are as follows:

E_α (Mev)	Cross Section (mb) (α, pn or d)	(α, p2n or t)	(α, p3n)	(α, αn)	(α, 2p)
23.2	...	≤ 0.4	*	...	...
26.5	...	3.7	*	...	...
29.4	≥ 5.8	18.4	*	$0.06^{+1}_{-0.06}$	...
32.3	...	...	...	4.4	...
35.9	...	...	...	18.8	0.2 ± 0.2
42.2	≥ 22.2	36.0	20 ± 20	29.7	...

* Bombardment energy below threshold for reaction.

Further experiments are planned, including the bombardment of thorium foil to produce large amounts of the long-lived uranium isotopes which are the (α, xn) reaction products. Foil bombardments are also planned to produce large amounts of fission products so that they may be studied by the method of aliquots.

The present results are interesting because cross sections for reactions involving the emission of charged particles are around 20 millibarns, the same order of magnitude as similar cross sections for target isotopes of the much heavier elements, neptunium and plutonium.

SPALLATION OF URANIUM-235 WITH 10-TO 25-Mev DEUTERONS

Richard M. Lessler

Uranium-235 has been bombarded with deuterons, and from analysis of the product yields the following preliminary cross sections have been obtained.

E_d (Mev)	Cross Section (mb) (d, n)	(d, 2n)*	(d, 3n)	(d, 4n)
8.8	1.86	2.69	...	
11.8	4.88	11.7	0.535	
14.8	7.62	17.5	10.3	
17.8	6.53	...	13.5	
20.8	7.2	15.5	16.3	0.405
23.8	7.54	...	8.30	2.36
23.8	6.77	12.6	9.58	4.08

* The (d, 2n) cross sections are upper limits, since the assumption was made that the proportional counter activity remaining on the sample plate after the decay of Np^{236}(22 hr), Np^{234}(4.4 day), and Np^{233}(35 min) was due to Np^{235}(410 days).

Proportional counter efficiencies were assumed to be 50% for evaporated samples and 70% for electroplated samples.

Work on this problem is continuing.

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NEW ISOTOPES ERBIUM-172 AND THULIUM-172

David R. Nethaway and Maynard C. Michel

In collaboration with Walter E. Nervik of the Livermore Laboratory, two new isotopes Er^{172} and Tm^{172} have been produced and characterized.

The primary purpose was to establish the mass number and half life of Tm^{172} and its parent. Tm^{172} had been produced by high-energy spallation of uranium, but only in low yield. In order to obtain sufficient activity, 18 mg of Er_2O_3 were irradiated in the MTR for 3 days, yielding by successive captures and beta decays the following isotopes (among others): Er^{171}(7.5 hr), Tm^{171}(1.9 yr), Er^{165}(10 hr), Er^{172}(?), and Tm^{172}(?). It was believed that the new activity was mass 172, and thus would be formed by a second-order capture process, probably in sufficient yield for mass separation.

A small amount of the gross sample was separated in the time-of-flight isotope separator in a search for any active Er isotopes present. Collections were made at masses 165, 171, and 172. The activities obtained were ~10 hr and 7.5 hr at masses 165 and 171, verifying the previous assignments, with no activity detected at mass 172. Since only 0.1% of the total sample was taken, this second-order capture product was not expected in this separation. Upon chemical separation of the thulium fraction, collection at masses 172 and 171 were made, giving activities of half life 63 ± 1 hr and something approaching the 1.9 yr previously reported for Tm^{171}. Successive milkings of the Er fraction yielded a half life of 49.8 ± 1 for the Er^{172} parent.

Brief studies with a NaI(Tl) scintillation spectrometer indicated prominent gamma rays at 1.79, 1.44, 1.09, 0.40, 0.18, and 0.076 Mev, and K x-rays at 0.049 Mev for Tm^{172}, as well as an approximately 1.5-Mev β^- particle.

A more complete description of this work is contained in Nethaway, Michel, and Nervik, New Isotopes: Er^{172} and Tm^{172}, UCRL-3286, Feb. 1956.

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NEUTRON-DEFICIENT ISOTOPES OF ANTIMONY

Ann Rhodes and John Rasmussen

Light isotopes of antimony have been made by alpha bombardment of indium in the 60-inch cyclotron.

Natural indium (95.8% In^{115}) in the form of In_2O_3 was bombarded with 49-Mev alpha particles. After chemical purification the antimony fraction showed the following peaks in the 50-channel gamma analyzer.

Energy (kev)	Apparent half life
90 ± 10	60 min
120 ± 10	73 min
156 ± 5	2.5 hr
(245) ± 5	··· (very weak)
385 ± 15	75 min
510 ± 15	80 min (annihilation radiation)

Antimony obtained by bombardment of In containing 65.4% In^{113} with 25-Mev alphas showed the 156-kev line, and also the 245-kev line with a half life of 2.5 hrs. The 156-kev line, and most probably the 245-kev line, belong to Sb^{117}. It seems likely that the remainder belongs to Sb^{116}, which is reported as having a 60-minute half life. The relative intensities of these Sb^{116} gammas are:

Energy (kev)	Relative Intensity
90	20
120	25
385	27
510	84 (annihilation radiation)

There is some evidence of a shorter half life present in the Sb fraction (~40 min), but at present there are not sufficient data to tell whether or not this is the as-yet-undiscovered Sb^{115}.

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TRITIUM PRODUCTION

Richard A. Glass, J. Gonzalez-Vidal, Glenn T. Seaborg, and W. H. Wade

An apparatus for tritium extraction from cyclotron and linear-accelerator targets and also a counting system for the measurement of tritium activities have been finished and calibrated. Bombardment of 10-mil U^{238} targets with 30-Mev protons, 20-Mev deuterons, and 40-Mev alpha particles has yielded preliminary average cross sections of 14.9 mb, 33.4 mb, and 2.2 mb.

Excitation-function determinations are now under way.

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CHEMICAL ENGINEERING (PROCESS CHEMISTRY)

NOTES ON WORK IN PROGRESS

Correlation of Limiting Current Density at Horizontal Electrodes under Free Convection Conditions

Eugene J. Fenech and Charles W. Tobias

Attempts at correlation of limiting current data by means of dimensionless equations have been encouraging. To date, the best correlation has been given by the equation

$$Nu' = 1.43\ (Sc\ Gr)^{1/4}$$

A technical report is now in preparation.

Stability of Perforated-Plate Trays

R. S. Brown, Donald N. Hanson, and C. R. Wilke

A limited amount of dry-plate pressure-drop data has been taken which is in complete agreement with data reported by Charles Hunt (UCRL-2696).

Stability tests using the air-water system are now in progress.

Thermal Conductivity of Gases at High Temperatures

Henry Cheung and C. R. Wilke

Experimental thermal conductivity obtained for N_2 at 104°C is in good agreement with literature values. Measurements for mixtures are in progress.

Gas-Liquid Partition Chromatography

Robert H. Houston and C. R. Wilke

A simple model theory for a gas-liquid partition chromatogram is about complete. The expression is derived from a differential equation giving the location of a component peak at any time as well as giving the variation of the maximum concentration with the column length, or retention time. An expression for the eventual constant rate of travel of the peak concentration has also been developed.

Experimental runs will be conducted to verify the theory.

The Effect of Pressure on Mass Transfer in the Gas Phase

Robert J. Fallat and C. R. Wilke

Vapor pressure and mass transfer data had been collected for the system naphthalene-air. Additional data with the system naphthalene-helium brought out an unforeseen difficulty in the measurements, making it necessary to collect further data with the two systems in order to obtain a more reliable correlation of the results. This latter work is now in progress.

Coalescence Rates in Agitation of Immiscible Liquids

J. H. Vanderveen and T. Vermeulen

The photocell assembly has been wired into continuous recording equipment, and calibration is in progress preparatory to actual measurements.

GENERAL CHEMISTRY

Robert E. Connick in charge

METALS AND HIGH-TEMPERATURE THERMODYNAMICS

Leo Brewer, William Hicks, Geoffrey James, and Frank Greene

Absolute f-Value Determination

The apparatus has been largely completed by the shops. We expect to make our first measurements during this next quarter. We have made a survey of the performance of various light sources that can be used in this work.

Ground State of C_2

The measurements of temperature coefficients of the Swan and Phillips emission bands of C_2 require rather large corrections for self-absorption, and it appears that absorption experiments will be necessary to fix the relative energy positions of the lowest singlet and triplet states.

Stability of Refractory Silicides

A tabulation of the best thermodynamic data available for the refractory silicides has been presented in UCRL-3352.

BASIC CHEMISTRY

Howard Cady, Robert Connick, Claude Coppel, Ladd Griffith, Robert Nickerson, Richard Poulson, Kenneth Pitzer, Robert Wood, and Earl Worden

Acetaldehyde t_1

Acetaldehyde d_1 was prepared by the same method as the acetaldehyde t_1 to determine if the location of the tritium on the aldehyde carbon is specific. The infrared spectrum indicated that the location of the deuterium by the reaction is about 90% efficient. The mass spectrum of the compound indicated about 5% of acetaldehyde d_2 was present. The mass spectrum is to be run again at a lower ionization potential where the parent peaks may be analyzed more easily.

The ultraviolet spectrum of the deutero compound was taken and compared with the spectrum of normal acetaldehyde. The structure overlying the general absorption was different for the two compounds, but the general absorption appeared to be the same. The deutero compound has a lower extinction coefficient throughout the region of absorption. This may be due to the presence of impurities, but requires more investigation.

Composition of Carbide Vapors

Revision of the induction-heated graphite cell for producing vapor from SiC appears to have eliminated condensation on the effusion lid, so a closer approach to equilibrium is indicated; additional study of the gas pressure has been done. The apparatus for condensing the vapors is being revised to give better cooling of the cold target.

Radiation-Induced Reactions in Nonaqueous Solutions

This work has been completed and a paper is being written.

Ruthenium Chemistry

Ruthenium tetroxide can be reduced to Ru(II) in perchloric acid, by use of Ti^{+3} as a reducing agent. The reaction is very complicated and is not quantitative. At least two species of ruthenium are formed. One is a nearly colorless species (350 to 800 mμ), perhaps Ru(III). The other species, Ru(II), has an absorption peak at 383 mμ with a minimum molar extinction coefficient of 2400.

An experiment similar to the above was performed, except that Cr^{++} was used as the reducing agent. In this experiment there was no evidence for the formation of the Ru(II) species. The lack of Ru(II) species can be explained by assuming the formation of a Cr-O-Ru complex that is slow to dissociate.

Kinetics of Rapid Reactions

The preliminary work on the new baffle, which was discussed in the preceding report (UCRL-3351), was extended somewhat. A high-speed-photography apparatus was made available at the Richmond Engineering Field Station. With the aid of this apparatus the actual firing of the mixer was seen, and from these photographs it was learned that there is very little or no cavitation behind the turbulator plate as it rises through the solution. Although this result did not substantiate our earlier views on cavitation, it did not contradict any experimental observations previously made. The results also gave an independent method of measuring the firing time, which was in approximate agreement with our earlier spectroscopic measurement.

With the preliminary work completed, the mixer is now being applied to the study of reaction rates. The present work is an attempt to check some rather critical results on the ferric-thiocyanate complexing reaction, which was studied earlier by John Below.

Plans are also being made to study several other reactions of two possible types; (1) complexing reactions, such as the one cited above, and (2) oxidation-reduction reactions (e.g., the argentic-ferrous system).

Analysis of Aqueous Ferrate (VI) Solutions

Analytical methods to check for the presence of lower oxidation states of iron in aqueous solutions of K_2FeO_4 are being developed.

As (III) in 1 N to 10 N NaOH does not give reproducible results as a reducing agent.

I^- in 0.6 N to 10 N NaOH gave results 4% to 5% low in oxidizing power. This seems to be due to a fault in the method and not to the presence of lower oxidation states of iron.

Measurement of the volume of oxygen evolved when H_2SO_4 is added to an aqueous solution of ferrate is now in progress. Preliminary results check within 1%. Solutions of I^- at pH 10 will also be tried as a reducing agent.

Determination of the Molecular Structure of Aluminum Hydride

The method previously described for adapting the Varian nuclear induction equipment for low-temperature experiments was to insert a single coil, as part of a balanced bridge, into a special dewar and cool with a precooled gas. Experiments have shown, however, that the bridge technique gave much less signal--about 40% of the signal given by the nuclear induction, all other conditions being equal. Since aluminum hydride, which is to be studied by using nuclear magnetic resonance, gives a very poor proton signal-to-noise ratio, it is important to obtain the maximum signal available, and for this reason extra effort is to be made to construct a nuclear induction probe for use at low temperatures.

Pure Quadrupole Resonance in Chloropentamine Cobalt(III) Chloride

So far, attempts to detect the quadrupole coupling of chlorine in cobaltic amine chloride complexes have been unsuccessful. The amine has been searched from 1 megacycle to 30 megacycles without detection of any resonance. Attempts will be made to use larger samples, in the hope that the lack of detection will prove due to insensitivity of the apparatus.

Chemical Shifts in the F^{19} Nuclear Magnetic Resonance in AlF_3^{3-n} Complexes

Work has been resumed on measurement of the nuclear magnetic resonance of fluoride ion in the aluminum fluoride complexes in order to determine something about the bonding of the fluorine to the aluminum. The chemical shift for the AlF^{+2} complex is about what is expected from the electronegativity of aluminum.

The chemical shifts of AlF_2^+ and AlF_3 indicate a somewhat decreased magnetic shielding of the F^{19} nucleus.

Crystal Structure of CrF_2

Chromous fluoride is being prepared by reaction of chromous chloride and gaseous anhydrous hydrogen fluoride. An x-ray diffraction pattern is to be sought from the powder.