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CHEMISTRY DIVISION QUARTERLY REPORT

June, July, August 1957

BERKELEY, CALIFORNIA

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June, July, August 1957

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

September 24, 1957

BIO-ORGANIC CHEMISTRY

M. Calvin, Director
Edited by Edward L. Bennett

LIGHT-INDUCED ENERGY STORAGE IN GREEN PLANT MATERIAL

Gordon Tollin,* Power B. Sogo, and Melvin Calvin

Photoinduced electron spin resonance signals have been observed in isolated chloroplasts and other green plant materials with a growth time not affected by reducing the temperature to -140°C. The luminescence of these materials has also been investigated under a variety of conditions. The results of these studies have been shown to be consistent with a mechanism involving the recombination of electrons and holes trapped in a quasi-crystalline lattice. Some details of such a mechanism have been proposed which suggest the mode of entry of the light energy into the photosynthetic pathway.

This work is described in a paper by Tollin, Sogo, and Calvin, "Light-Induced Energy Storage in Particulate Material Derived from Green Plants," which was presented at the A.A.A.S. Symposium at Stanford University on August 28, 1957; it is being issued as UCRL-3942, Oct. 1957, in the form in which it was subsequently presented at the Gatlinburg Symposium on Photoperiodism, Gatlinburg, Tenn., October 1957, under the title "Energy Transfer in Ordered and Unordered Photochemical Systems."

*National Science Foundation Postdoctoral Fellow, 1956 to 1957.

UNSTABLE CO_2 FIXATION IN PHOTOSYNTHESIS

Helmut Metzner, Helmut Simon and Barbara Metzner

Studies comparing the effects of various killing procedures on the amount of the photosynthetic $C^{14}O_2$ fixation by algal cells had led to the conclusion that a certain part of the CO_2 fixed during the first seconds of photosynthesis is only unstably bound.[1,2] The following experiments were performed to measure the portion of the labile CO_2 fixation complex. To avoid losses during the preparation of the planchets, the total activity was determined by use of ionization chambers according to the method proposed by Wilzbach and Sykes.[3]

After assimilating radioactive CO_2 for periods between 5 seconds and 5 minutes the algae (Scenedesmus obliquus) were killed by pouring the whole suspension into organic solvents, in most cases acetone cooled down to temperatures between -30 and -40°C. Control experiments were performed in the usual way, killing the algae by boiling ethanol. In some series that were planned to check the influence of hydroxylamine on the CO_2 fixation rate,[1,2] a solution of hydroxylamine hydrochloride in distilled water (final concentration 10^{-2} M) was added immediately (< 2 seconds) before the killing. One ml of glacial acetic acid was added to the killed algae to remove excess bicarbonate. Then an aliquot of 100 λ was taken by precooled micropipettes and injected into a thick-walled glass tube containing copper–copper oxide mixture and some glucose. The sample was quickly frozen on the bottom of the vessel, which was precooled in a vessel of liquid oxygen. The bomb was placed in a constant-temperature bath and warmed to -45°C to thaw the CO_2. The CO_2 was removed in vacuo by evacuating the bomb tube for an exactly determined time.

After sealing, the glass tube and contents were heated to 650°C; by this means all organic material was burned to CO_2. The bomb was cooled to room temperature and broken inside a vacuum line. Gases were fractionated by a temperature gradient and the carbon dioxide was transferred into an ionization chamber. The radioactivity of the carbon dioxide was measured by a vibrating-reed electrometer. Figures obtained in this way were taken as values for the "total fixation."

To determine the "stable fixation," another aliquot portion of the algal suspension was spread on an aluminum planchet or on a thin Mylar foil which was inserted into another bomb and burned in close contact with a copper–copper oxide mixture and some glucose as carrier. In some experiments a small amount of potassium perchlorate was added to this mixture to secure a complete combusion of the organic material. This gave the figures for the "stable fixation."

---

[1] H. Metzner and B. Metzner, in Chemistry Division Quarterly Report, UCRL-3710, March 1957, p. 4.

[2] Metzner, Metzner, and Calvin, Arch. Biochem. and Biophys., in press.

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

In some experiments, the stable-fixation values were obtained by counting the planchets with a Geiger-Mueller tube. The estimation of a conversion factor enabled us to express the G-M tube measurements (counts/min) in the same units as the ionization-chamber figures (millivolts/min). It has to be emphasized, however, that these conversion factors are not very reproducible. The value depends upon the amount of algae spread over the surface of the planchet (self-absorption) and therefore changes with the concentration of the algal suspension used for the particular experiment. For this reason it seems necessary to burn both the original suspension and the planchet to get reliable figures on the "total" and "stable" fixation. The "unstable fixation" may be given as the difference between these two values.

In agreement with our earlier findings, we found in all cases that the algae showed a higher total C^{14} fixation after killing with cold acetone or ethanol than after killing with boiling alcohol. Table I demonstrates that the quantity of the unstable CO_2 fixation complex is negligible after hot ethanol killing, whereas both cold acetone and cold ethanol preserve a portion which accounts for at least 30% of the total C^{14} fixation.

Table I

Total, stable, and unstable CO_2 fixation, measured after different killing procedures

(5 sec photosynthesis after 90 sec flushing with N_2 in the light)

Experiment number	Killing procedure	Total fixation (mv/min)[a]	Stable fixation (mv/min)[a]	Unstable fixation (mv/min)	Unstable fixation (rel. %)
12	acetone (-30°C)	32.9[b]	22.0	10.9[b]	33.1[b]
11	ethanol (-30°C)	37.5	26.2	11.3	30.1
11	ethanol (+78°C)	34.7	33.8	0.9	2.6

[a]Values are for the 100-λ aliquot.

[b]Value too small (time interval between killing and freezing of the sample, >60 sec).

The first experiments had to exclude the possibility that this additional fixation is due to excess radioactive bicarbonate. To check this some bombs were filled with a solution of bicarbonate in acidified acetone. It could be demonstrated that after the evacuation procedure used in our tests no activity was found. On the other hand, a small fixation was observed when the bicarbonate solution was added to algae previously killed by acetone. In this case one sample was taken as soon as possible after the addition of the acid (< 45 seconds), two others after 4 and 8 minutes. Table II shows that the amount of unstably bound C^{14} is constant over a period of 8 minutes. This is quite different from the behavior of the labile complex formed in living cells (see below).

Table II

CO_2 fixation by dead algal cells

Sample	Total fixation (mv/min)	Stable fixation (mv/min)	Unstable fixation absolute (mv/min)	Unstable fixation relative (%)
after 45 sec	4.3	0.0	4.3	100
after 4 min	4.9	0.0	4.9	100
after 8 min	4.0	0.0	4.0	100

It is possible that this portion of unstable fixed CO_2 is due to an unspecific binding of the carbon dioxide to proteins or to buffer systems inside the cells. The observed value of about 4.4 mv/min was added to the measured background of the ionization chamber so that it was automatically subtracted in all experiments with living cells. In addition, experiments on dark fixation under anaerobic conditions showed negligibly low values for the CO_2 fixation ($<$ 1 mv/min). This is a good indication that even in living cells there is no unspecific binding of CO_2 that could account for the observed unstable carbon dioxide fixation.

The amount of the labile CO_2 complex depends to a certain degree upon the pretreatment of the algae. Flushing the suspensions with CO_2 until the time of addition of the radioactive bicarbonate leads to a much smaller total fixation (dilution effect), but the relative amount of the unstably bound CO_2 is higher than in control experiments in which before the experiment itself the algae were flushed (90 seconds) with pure nitrogen. In all the following experiments the suspensions were pretreated with nitrogen. Therefore we are not dealing with steady-state conditions, but the fixation rate is greatly increased--which makes the figures more reliable.

To obtain an estimate of the absolute amounts of the labile complex, algae were allowed to photosynthesize for periods between 5 seconds and 5 minutes. Table III gives the values for the C^{14} fixation. The amount of the labile compound(s) increases much more slowly than the total fixation, up to 2 minutes. After about 1 to 2 minutes a certain saturation value seems to be reached. If the photosynthesis is extended to 5 minutes, however, there is a new increase in the amount of the unstable complex. This result is reproducible. We have to assume that after longer photosynthesis periods another compound is formed which is partially lost during the preparation and counting of the planchets. This may be glycolic acid, which is present in fairly high amounts when the suspensions are concentrated at low temperatures.

Table IV gives some data for a 7.5 second photosynthesis experiment. It demonstrates the stability of the labile complex. After addition of acetic acid a first sample was taken as soon as possible ($<$ 45 seconds), then further samples after the suspension had stood at -30°C for a period of 16 minutes. The relative values for the labile fixation complex decrease to

about 20%. This figure was obtained in several experiments. This suggests that we actually are dealing with at least two unstable compounds (besides the glycolic acid). The data indicate that 50% of the most unstable product splits within 4 minutes. It requires at least 30 seconds to inject the first sample into the bomb. In addition to this, upon mixture of the algal suspension with the cold solvents the temperature of the whole system increases to about -10°C before it can be recooled. Therefore we are forced to conclude that all the figures for the absolute and relative amounts of unstable fixation in our tables are minimum values. Very careful measurements actually indicated that after 5 seconds of photosynthesis more than 90% of the radioactive carbon is unstably fixed.

Table III

Kinetics of the CO_2 fixation

(photosynthesis after 90 sec flushing with N_2 with light)

Duration of photosynthesis	Total fixation (mv/min)	Stable fixation (mv/min)	Unstable fixation absolute (mv/min)	Unstable fixation relative (%)
5 seconds	27.7	16.7	11.0	39.7
30 seconds	241.2	209.1	32.1	13.3
1 minute	646.3	570.8	75.1	11.6
2 minutes	1417.7	1334.7	83.0	5.9
5 minutes	4263.4	3762.1	501.4	11.8

Table IV

Stability of the labile CO_2 fixation complex

(7.5 sec photosynthesis after 90 sec flushing with N_2 in the light)

Sample	Total fixation (mv/min)	Stable fixation (mv/min)	Unstable fixation absolute (mv/min)	Unstable fixation relative (%)
after 45 sec	26.9	15.6	11.3	42.0
after 4 min	22.2	15.6	6.6	29.7
after 8 min	21.4	15.6	5.8	27.1
after 16 min	19.6	15.6	4.0	20.4

Our values for the dark fixation of the carbon dioxide are only preliminary figures. Table V gives the amount of the CO_2 dark fixation after aerobic and anerobic pretreatment. As already mentioned, after darkened algae have been flushed with pure nitrogen there is practically no labile complex at all, whereas after a short oxygen flush there is a small but significant unstable fixation. Whether this leads to the same complex as the photosynthetic fixation has not yet been studied. After a short pre-illumination under anaerobic conditions we find a considerably increased CO_2 fixation in both the stable and the unstable intermediates. In the following dark period this capacity drops to about 1/3 to 1/4 of the original value in the course of 15 seconds. The similar behavior of stable and unstable fixation makes us believe that the labile CO_2 complex has a close connection with the photosynthetic CO_2 fixation. It may well be that this complex is a precursor of the stable intermediates that have thus far been identified.

Table V

Dark fixation of CO_2 without and after preillumination
(all samples killed with acetone, -30°C)

Experiment number	Pretreatment	Dark fixation	Total fixation (mv/min)	Stable fixation (mv/min)	Unstable fixation absolute (mv/min)	Unstable fixation relative (%)
11	70 min N_2/dark	30 sec	0.4	0.0	0.4	
12	10 min N_2/dark+ 3 min O_2/dark	60 sec	4.9	1.5	3.4	69.4
12	90 sec N_2/light	60 sec	24.3	12.1	12.2	50.2
12	90 sec N_2/light+ 15 sec dark	60 sec	7.2	4.3	2.9	40.3

Earlier experiments had demonstrated that the addition of hydroxylamine to the algae just before the killing increased the total fixation and decreased the loss during the following fractionation procedure of the suspension.[1,2] This looks like a "stabilization effect" caused by the NH_2OH. The very first experiment with the ionization chamber method confirmed this. After 5 seconds' photosynthesis 47% of the C^{14} was unstably bound, whereas after addition of NH_2OH this loss was completely prevented. Table VI gives another typical example. In addition to these figures, the studies of the kinetics of the carbon dioxide fixation in the NH_2OH experiments give comparable values (Table VII). In this case, too, there is a new increase of the unstably bound CO_2 after 5 minutes, indicating that this extra fixed product is not stabilized by the NH_2OH.

Table VI

Influence of hydroxylamine upon the total, stable, and unstable CO_2 fixation (5 sec photosynthesis after 90 sec flushing with N_2 in the light)

Experiment number	Killing procedure	Total fixation (mv/min)	Stable fixation (mv/min)	Unstable fixation absolute (mv/min)	Unstable fixation relative (%)
12	acetone (-30°C)	32.9	22.0	10.9	33.1
11	NH_2OH-acetone (-30°C)	38.5	38.1	0.4	1.0

Numerous experiments with NH_2OH demonstrated that this "stabilization effect" is not very reproducible. Sometimes a time interval of less than 2 seconds between the addition of the NH_2OH to the algae and the killing process is enough to stabilize the CO_2 complex completely. In other experiments, however, only a certain part is stabilized even if 10 seconds elapse between the addition of the hydroxylamine and the killing with cold acetone. This might be explained by the fact that the permeability of algal cells for NH_2OH is very low and changes in the physiological state of the algae may lead to different stabilization rates.

Table VII

Kinetics of the CO_2 fixation, measured after the addition of hydroxylamine (photosynthesis after 90 sec flushing with N_2 with light)

Photosynthesis	Total fixation (mv/min)	Stable fixation (mv/min)	Unstable fixation absolute (mv/min)	Unstable fixation relative (%)
5 seconds	17.6	19.4	(-)	(-)
10 seconds	95.6	94.0	1.6	1.7
1 minute	603.9	593.5	10.4	1.7
2 minutes	1389.8	1477.7	(-)	(-)
5 minutes	3975.1	3554.8	420.3	10.6

To study the effect of NH_2OH, a series of comparable experiments was performed in which the hydroxylamine was added either before or after the killing. Table VIII demonstrates that NH_2OH given before the cold organic solvents were added increases the total fixation. In all these tests the standard procedure was applied. Aliquots of the suspension were plated on aluminum planchets and the radioactivity was measured immediately with a G-M tube. The suspension was stored at -5°C and a second sample was taken after 2 hours. Subsequently the solution was warmed to room temperature and a third sample was removed after an additional 20 hours.

Table VIII

Influence of NH_2OH, added before or after killing, upon the total CO_2 fixation (5 sec photosynthesis after 90 sec flushing with N_2 in the light)

Killing procedure[a]	1st Measurement immediate plating[b]	2nd Measurement after 2 hr at -5°C[b]	3rd Measurement after 20 hr at +25°C[b]
Ethanol (+78°C)	281.6	284.4	278.4
Ethanol (-30°C)	500.5	407.9	405.0
NH_2OH-ethanol (-30°C)	671.8	579.2	591.9
NH_2OH-ethanol (+78°C)	535.6	472.7	468.3
Ethanol (-30°C)-after 5 sec NH_2OH	446.5	424.2	439.6
Ethanol (-30°C)-after 60 sec NH_2OH	444.9	416.0	437.1
Acetone (-30°C)	281.7	228.2	196.0
NH_2OH-acetone (-30°C)	501.5	283.7	327.9
Acetone (-30°C)-after 5 sec NH_2OH	317.3	258.9	297.0

[a]These experiments with ethanol and with acetone are quantitatively not comparable because different suspensions were used.

[b]Figures are 10^4 cpm/ml wet-packed cells.

In all cases there was a decrease in the activity during the first 2 hours except after the algae were killed with boiling ethanol. This procedure apparently destroyed the unstably bound CO_2 completely. While standing at room temperature for 20 hours, the pure ethanol extract of the algae lost an insignificant amount of unstably bound C^{14}, whereas the pure acetone extract lost significant radioactivity.

In all cases, however, in which NH_2OH was added either before or after the addition of the solvent, there was an increase of the total fixation. This indicates that a certain percentage of the complex, presumably the less unstable compound, is destroyed in the solution, but in the presence of NH_2OH a portion of it is transformed to a more stable product. Thus, the experiments listed in Table VIII support the suggestion made as a result of the first experiments that the hydroxylamine causes a certain stabilization of at least a part of the unstably bound CO_2. We hope to be able to chromatograph and identify the unknown fixation product in the form of this more stable derivative.

An unstable CO_2 fixation by living plant tissues, independent of the binding by carbonate and phosphate buffers inside the cells, was postulated long ago.[4,5] Shafer[6] could demonstrate that this CO_2 uptake is enhanced in the light. Our data, especially the lack of dark fixation under anaerobic conditions, show that this fixation cannot be an unspecific addition to basic groups of proteins[7] or other cell constituents; it must be a fixation that is at least partly light-dependent. The compound for which we have evidence cannot be identical either with the complex described by Ruben and his co-workers[8] or with the "primary product" postulated in some Russian publication,[9,10] because the product with which we are dealing would not have survived the killing and isolation procedures used by Ruben as well as by the Russian scientists. Our CO_2 fixation product cannot be identified with Warburg's "functional carbonic acid,"[11] which is CO_2 reversibly bound to the glutamic acid-γ-amino-butyric acid system,[12] because the end fixation product, glutamic acid, would be stable in cold acetone.

We used the most reliable data to calculate the pool size of the unstable fixation product. We got a value of 10 to 15 millimoles CO_2 per liter of chloroplastic material. This figure is higher than that given for the amount of ribulose diphosphate, which was thought to be the first CO_2 acceptor.[13] On the other hand, our value is of the same order of magnitude as the chlorophyll content.

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[4] S. Kostytschew, Ber. deut. botan. Ges. 39, 319 (1921).

[5] R. Willstätter and A. Stoll, Untersuchungen über die Assimilation der Kohlensäure (Springer-Verlag, Berlin, 1917).

[6] J. Shafer, Jr., Plant Physiol. 13, 141 (1938).

[7] M. Siegfried, Z. physiol. Chem. 44, 85 (1905); 46, 401 (1905).

[8] Ruben, Hassid and Kamen, Science 61, 661 (1939); Ruben, Kamen, and Hassid, J. Am. Chem. Soc. 62, 3443 (1940).

[9] E. A. Bbichenko and N. I. Zakharova, Biokhimiya (English translation) 21, 377 (1956).

[10] N. G. Doman, Biokhimiya (English translation) 21, 71 (1956).

[11] O. Warburg and G. Krippahl, Z. Naturforsch. 11b, 718 (1956).

[12] O. Warburg, H. Klotzsch and G. Krippahl, Z. Naturforsch. 12b, 266 (1957).

[13] M. Calvin and P. Massini, Experientia, 8, 445 (1952).

GLUTAMIC ACID-GLUTAMINE RELATIONSHIPS

Helmut Simon

This work was done in order to get some insight into and to explain the effects of diazo-oxo-norleucine (DON) inhibition upon the amino and the acids of the Krebs cycle. Specifically the relationship between glutamic acid, glutamine, and α-ketoglutaric acid was investigated. Barker et al. had already found in 1956 that azaserine acts during photosynthesis by Scenedesmus to produce a marked increase in levels of glutamine and the acids of the Krebs cycle accompanied by a corresponding depletion of the amino acids' reservoirs.[1]

Van der Meulen studied the influence of DON and azaserine on photosynthesis and on the amino acid and dicarboxylic acid pools of Chlorella and Scenedesmus.[2] On Scenedesmus the effects of azaserine and of DON are very much the same. No inhibition was found with azaserine on Chlorella.

Long-time experiments with DON showed a very sharp increase in the amount of glutamic acid formed during 5-minute photosynthesis. A very pronounced maximum is observed after 20 minutes of DON inhibition at a suitable concentration. After this time a very sharp drop occurs, and after 50 minutes the amount of glutamic acid drops to 20% of that after 20 minutes and remains then nearly constant. The amount of α-ketoglutaric acid and glutamine goes up all the time.

Van der Meulen compared also the specific activities of glutamic acid and glutamine. During the first 25 minutes after DON injection, glutamine seems to have a higher specific activity than glutamic acid. These facts lead one to suspect there may be a way by which glutamine can be formed other than through glutamic acid. One method of getting an answer is a comparison of the distribution of the radioactivity of the glutamic acid, α-ketoglutaric, and the glutamine after a photosynthetic experiment with C^{14}. The following experiments were done for this purpose.

Experimental Procedures

A. Counting Techniques and Degradation Studies on Glutamic Acid

All the radioactivity measurements for the determination of radioactivity distribution were made by measuring CO_2 in ionization chambers. The organic material was combusted in sealed tubes with CuO according to Wilzbach;[3] CO_2 from degradations was trapped in sodium hydroxide solution and converted to $BaCO_3$. The CO_2 from $BaCO_3$ was liberated by $CuSO_4$ at 650°C in a sealed tube and transferred into an ionization chamber.[4]

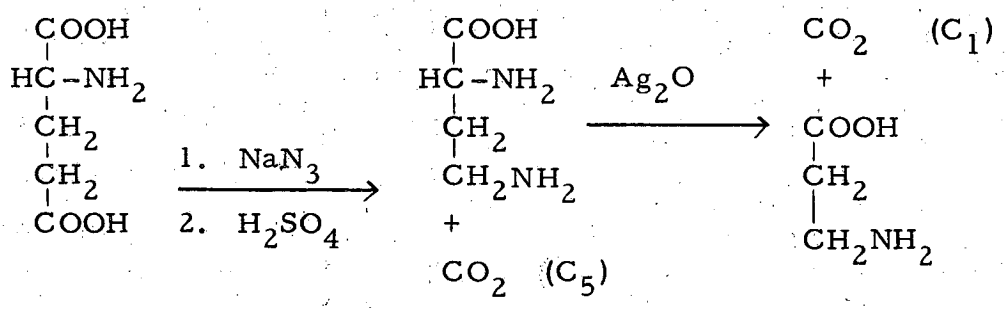
[1] Barker, Bassham, Calvin, and Quarck, J. Am. Chem. Soc. 78, 4632 (1956).

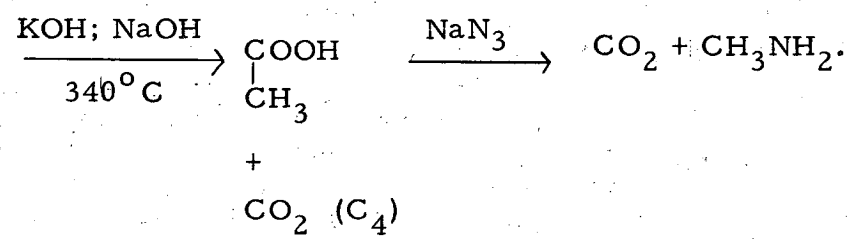
[2] P. Y. F. van der Meulen, in Chemistry Division Quarterly Report, UCRL-3710, March 1957, p. 29.

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

[4] Whittemore, Ludwigsen, and Tolbert, in Chemistry Division Quarterly Report, UCRL-3595, Oct. 1956, p. 12.

For the degradation of glutamic acid the following procedure was tried:[5]





The Schmidt degradation was checked with glutamic acid-5-C^{14} and glutamic acid-1-C^{14}. The general procedure is described by Phares.[6] To a mixture of 2 mM glutamic acid (294.3 mg) and 200 mg sodium azide was added 1.2 ml sulfuric acid (100%) at 10°C. During 10 minutes the temperature was raised to 70°C and then was maintained for 30 minutes. The usual precautions were taken to avoid any atmospheric CO_2. The yield of CO_2 is about 60% to 70%. Three determinations of the radioactivity in millivolts per minute per millimole of the glutamic acid-5-C^{14} and the $BaCO_3$ derived from it by the Schmidt reaction were as follows:

	Sample number	Activity (mv/min/mM)
Glutamic acid	1	1939
	2	1946
	3	1952
	average	1946
$BaCO_3$	1	1860
	2	1839
	3	1853
	average	1871

[5] Wang, Christiansen, and Cheldelin, J. Biol. Chem. 201, 683 (1953).

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

Calvin

The $BaCO_3$ has only 95.1% of the activity of the glutamic acid. There were three possible reasons for this result: (a) a blank of $BaCO_3$; (b) an incomplete conversion of $BaCO_3$ to CO_2 in the sealed tubes, and (c) the possibility that, at least for glutamic acid, some of C_1 is split off by the Schmidt procedure. $BaCO_3$ obtained from glutamic acid-1-C^{14} by a Schmidt degradation had about 6% of the glutamic acid-1-C^{14} radioactivity. This shows clearly that some CO_2 is derived from C_1 of glutamic acid by this technique.

An experiment was done to check the oxidative decarboxylation of α, γ diaminobutyric acid-1-C^{14} to β-alanine. It was found that about 40% of the CO_2 is derived from further oxidations. The specific activity of the CO_2 that was trapped during the reaction of α, γ diaminobutyric acid-1-C^{14} with silver oxide was only 58.4% of the theoretical amount. Therefore, the C_1 was determined by ninhydrin oxidation. The yields of the reaction from β-alanine to acetic acid were generally poor, usually only 5% to 10%.

B. Isolation of Glutamic Acid and Glutamine from Photosynthesis or Dark-Fixation Experiments

The algae were killed by pouring the suspension into 4 parts of boiling ethanol. This suspension was immediately cooled to 5°C. The insoluble portions were separated by centrifugation and extracted with cold 20% ethanol. The combined extracts were concentrated by freeze drying. The concentrated extracts of 200 λ wet packed cells were put on one sheet of Whatman No. 3 paper for two-dimensional chromatographic separation. For the first direction a water-saturated phenol was used. The pH of this was adjusted to pH 5.5 with 0.1 M NaOH. In the box was a 0.3% ammonia solution. For the second direction butanol–propionic acid–water was used.

The glutamine and glutamic acid were eluted. The glutamine was hydrolyzed by heating with dilute HCl for 5 hours and rechromatographed in one direction with phenol. After dilution the glutamic acid was recrystallized twice. The specific activity per mM of both these samples of glutamic acid (one derived from glutamine) was very carefully determined and compared with the $BaCO_3$ after the Schmidt reaction and ninhydrin oxidation.

C. First Photosynthesis Experiment

To 40 ml 2% Chlorella suspension (flask shaken) was added 0.8 ml 2.5×10^{-3} M K_2HPO_4/KH_2PO_4 buffer* followed by 15 min flushing with a mixture of 3.7% CO_2 in air in the light of two photoflood bulbs. After this time a solution of DON was injected (10 μg/ml suspension) and, after 15 additional minutes in the light and with CO_2, 1.5 ml $NaHCO_3$ was added (0.026 M, 400 μC/ml). The lollipop was shaken for 15 minutes in the light, then the algae were killed. The degradation studies showed:

Glutamic acid	C_1 = 21.2% of total activity
	C_5 = 22.7% of total activity
Glutamine	C_1 = 19.6% of total activity
	C_5 = 20.2% of total activity

*In all the experiments a volume of this buffer was added equal to the volume of the wet packed algae used.

The ratio of glutamic acid to glutamine on the paper was about 1:1. In order to find out whether glutamic acid and glutamine were derived from different sources it was necessary to shorten the photosynthesis times to avoid a uniform distribution of radioactivity. Therefore, the activity of C_1 and C_5 of glutamic acid of ordinary photosynthesis experiments of varying times was determined. These experiments were done in the usual way.

D. Second Photosynthesis Experiment

A Chlorella suspension was kept 15 min in the light and flushed with 0.6% CO_2 in air. Then the $HC^{14}O_3^-$ was injected. The results of the glutamic acid degradations are as follows:

Photosynthesis Time (min.)	Glutamic acid C_1 (% of total activity)	Glutamic acid C_5 (% of total activity)
10	19.7	22.5
6	21.3	23.0
3	21.7	24.2
1	32.0	12.0

The values of the 1-min photosynthesis are not very accurate. The activities were very low. The error may be as high as 15% to 20%.

It thus appears, as a result of these studies, that even in as short a time as 3 min the glutamic acid is nearly uniformly labeled. Nevertheless, the total amount of radioactivity in glutamic acid is rather small (roughly 0.3% of the total fixation).

It is not surprising that glutamic acid formed in the light is very soon uniformly labeled if one assumes that it is formed via the Krebs cycle from acetate derived from the same source as the C_1-C_2 and C_5-C_6 pairs of hexose.

Quite another route is expected from dark fixation. The glutamic acid from Scenedesmus after 10-min dark fixation showed the following distribution: C_1, 96%; and C_5, 8% (5% of C_1 is found in C_5 as mentioned above).

E. Experiments With Photosynthesis Together With Dark Fixation

Preliminary Experiment. 25 ml of 2% Chlorella suspension + 0.5 ml phosphate buffer; 2 hr in complete darkness, the last 10 min with 25 μg DON/ml suspension; 3 min light and 0.5% CO_2 in air; 10 min dark fixation with $HC^{14}O_3^-$; 1 min light; and again 5 min dark fixation. Glutamic acid was about 16% of the total fixation, but none or only very little glutamine-C^{14} was found. (The ATP pool for amidation of carboxyl group may be too small.) This was also done in a further series of experiments (see Table IX).

Six experiments were carried out to determine the fixation of $C^{14}O_2$ into some amino acids and related compounds with and without DON and under varying fixation conditions. The experimental conditions and results are summarized in Table IX.

The distribution of C^{14} in glutamine and glutamic acid from mixed photosynthesis and dark-fixation experiments was determined. After 1 min photosynthesis and 10 min dark fixation, the label was not far from a uniform distribution: glutamic acid: C_1, 25.3; C_5, 24.6; glutamine: C_1, 24.5; C_5, 23.9. Therefore, the times were further shortened.

The experimental conditions and the relative fixation of C^{14} into different compounds are shown in Table X for these even shorter-time experiments. In some of these experiments small amounts of NH_4NO_3 were added approximately 5 min before the radioactive bicarbonate was added. Table XI summarizes the degradation data obtained from these experiments.

Results and Discussion

In the light, glutamic acid is uniformly labeled very rapidly. That is in accordance with its formation from hexose (or PGA) via acetate and the Krebs cycle. In the dark, the glutamic acid may be formed by carboxylation of γ-aminobutyric acid and(or) by carboxylation of phosphoenol pyruvate and thence via the Krebs cycle. Labeled γ-aminobutyric acid was found, for instance, in Expt. 7.

In experiments inhibited with DON, glutamic acid seems to be obtained from glutamine (Expts. 7 and 9). The reductive amination of α-ketoglutaric acid appears blocked (Expt. 9). In noninhibited experiments the main route to glutamic acid is via α-ketoglutaric acid, but glutamine is, at least in the presence of ammonia, formed by another route (compare Expts. 8 and 9). It turned out that NH_4^+ ions have the same general effect as DON. The effects are very pronounced in the presence of DON and NH_4^+ (Table X).

Another effect of DON seems to be to block reductive transamination and amination reactions that are pyridoxal-dependent. Therefore, the ammonia pool is built up and all nonreductive reactions that use ammonia and are pyridoxal-independent are favored. Therefore, perhaps an amidation of α-ketoglutaric acid takes place. This amide may form a heterocyclic compound I (Figs. 1 and 2).

Another sink for NH_3 would be the amidation of α-aminobutyric acid (formed by decarboxylation of glutamic acid) to its amide. Both these amides can lead to glutamine. Compound III, by direct α carboxylation, would produce C_1-labeled glutamine. Compound I, by reduction and amiolysis, would lead to glutamine from α-ketoglutaric without passing through glutamic acid. This latter reaction would also account for the primary entry of nitrogen into the amino group of glutamine without passing through glutamic acid.

Table IX

Effect of DON upon the distribution of C^{14} in Chlorella after various fixation conditions[a]

	Experiment number					
	1	2	3	4	5	6
Conc. of DON (μg/ml)	15	0	15	0	15	0
cpm on paper	1.4×10^6	4.6×10^5	1.4×10^5	7.3×10^4	4.8×10^5	5.5×10^5
Compound	Percentage distribution[b]					
Glycolic acid	24	20				
Glycine and serine	2.3	6.3	3.2	7.4	3.9	5.3
Alanine	0.45	1.3	2.5	1.6	2.9	4.1
Glutamine	0.25	< 0.1	0.0	0.0	0.2	0.3
Glutamic acid	0.25		8	5.1	3	
Aspartic acid	0.55	1.7	13	12.5	4.1	4.6
Malic acid	1	1.5	1.1	2	0.9	0.6

[a]Experimental conditions

Expt. 1: 10 ml of 1.5% Chlorella suspension--120 min in the dark in distilled water, 15.0 μg DON/ml suspension; 20 min light, flushed with 0.5% CO_2 in air; 6 min photosynthesis with 200 λ $HC^{14}O_3^-$.

Expt. 2: As in Expt. 1, without DON. Algae were kept for 150 min in the dark.

Expt. 3: 10 ml of 1.5% Chlorella suspension--180 min in the dark in distilled water, 15.0 μg DON/ml suspension; 20 min dark; 2 min light; 15 min dark fixation with 200 λ $HC^{14}O_3^-$.

Expt. 4: As in Expt. 3 without DON. Algae were kept 120 min in the dark.

Expt. 5: 10 ml of 1.5% Chlorella suspension--240 min in dark in distilled water, 15.0 μg DON/ml suspension; 15 min dark; 3 min light with 0.5% CO_2 in air; 1 min photosynthesis; 10 min dark fixation with 200 λ $HC^{14}O_3^-$.

Expt. 6: As in Expt. 5 without DON. The algae were kept 275 min in the dark.

[b]The % distribution is based upon the amount of C^{14} placed on the origin of the chromatogram.

Table X

Effect of DON and(or) NH_4NO_3 upon the distribution of C^{14} in Chlorella after various fixation conditions.[a]

	Experiment Number			
	8	9	10	11
	NH_4NO_3	NH_4NO_3 and DON		DON
cpm on paper	3×10^5	2.6×10^5	2.5×10^5	1.7×10^5
	Percentage distribution			
Compound				
α-Ketoglutaric acid	...	3.3	...	0.22
Glutamic acid	12	9.2	10	11.0
Citric acid	1.6	4.3	0.56	1.9
Malic acid	8.9	31.6	4.1	16.5
Fumaric acid	2.8	8	1.2	4.1
Glycine and serine	3.8	1.7	2.5	2.6
Aspartic acid	28	3.8	29	21
Alanine	14.5	5.7	20	20
Glutamine (citrulline?)	1.1	3.1	<0.1	<0.1

[a] Experimental Conditions

Expt. 7: 10 ml 2% Chlorella suspension--11 min in light with 0.6% CO_2 in air, 35 μg DON/ml suspension; 15 min light with 0.6% CO_2 in air, 300 λ $HC^{14}O_3^-$; 30 sec photosynthesis; and 6 min dark fixation.

Expt. 8: 10 ml of 2% Chlorella suspension--15 min in light with 1.0% CO_2 in air, NH_4NO_3 added to make a 10^{-3} M solution; 5 min light with 1.0% CO_2 in air; 15 sec photosynthesis, 4 min dark fixation with 300 λ $HC^{14}O_3^-$.

Expt. 9: 10 ml of 2% Chlorella suspension--5 min in light with 1% CO_2 in air, 35 μg DON/ml suspension added; 10 min light with 1% CO_2 in air, NH_4NO_3 added to make 10^{-3} M solution; 5 min light with 1% CO_2 in air, 15 sec photosynthesis; and 4 min dark fixation with 300 λ $HC^{14}O_3^-$.

Expt. 10: 10 ml of a 2% Chlorella suspension--20 min in light with 1% CO_2 in air; 15 sec photosynthesis; and 4 min dark fixation with 300 λ $HC^{14}O_3^-$.

Expt. 11: 10 ml of a 2% Chlorella suspension--5 min in light with 1% CO_2 in air, 35 μg DON/ml suspension added; 15 min in light with 1% CO_2 in air; 15 sec photosynthesis; and 4 min dark fixation with 300 λ $HC^{14}O_3^-$.

Table XI

Distribution of C^{14} in glutamine, glutamic acid, and α-ketoglutaric after short-time fixation.*

		Experiment number		
		7	8	9
		DON	NH_4NO_3	NH_4NO_3 + DON
Compound	Position	% Distirbution of activity		
Glutamic acid	C_1	40.7; 40.2	19.9	54.1
	C_5	20.4		12.9
Glutamine	C_1	37.4	61.5	49.7
	C_5	22.9		11.0
α-Ketoglutaric	C_1			15.7

*For experimental conditions, see Table X.

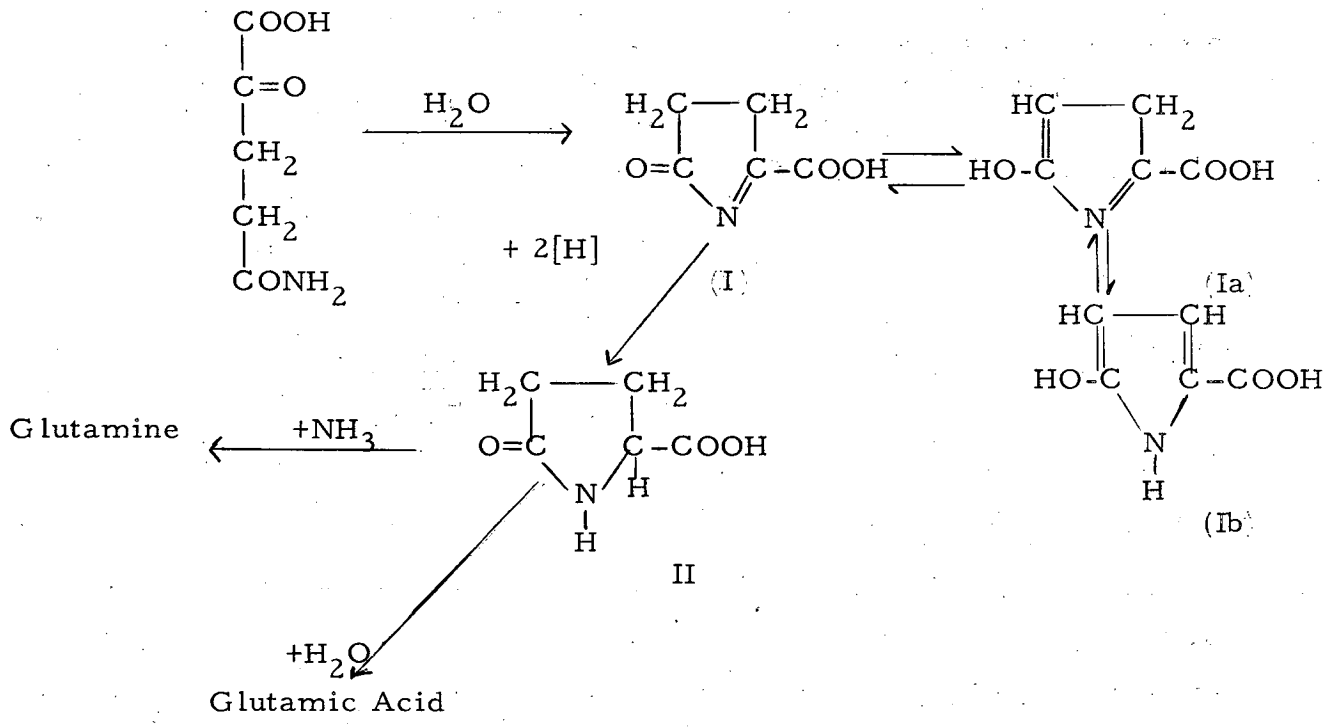


Fig. 1. A route from glutamine to glutamic acid not involving glutamic acid.

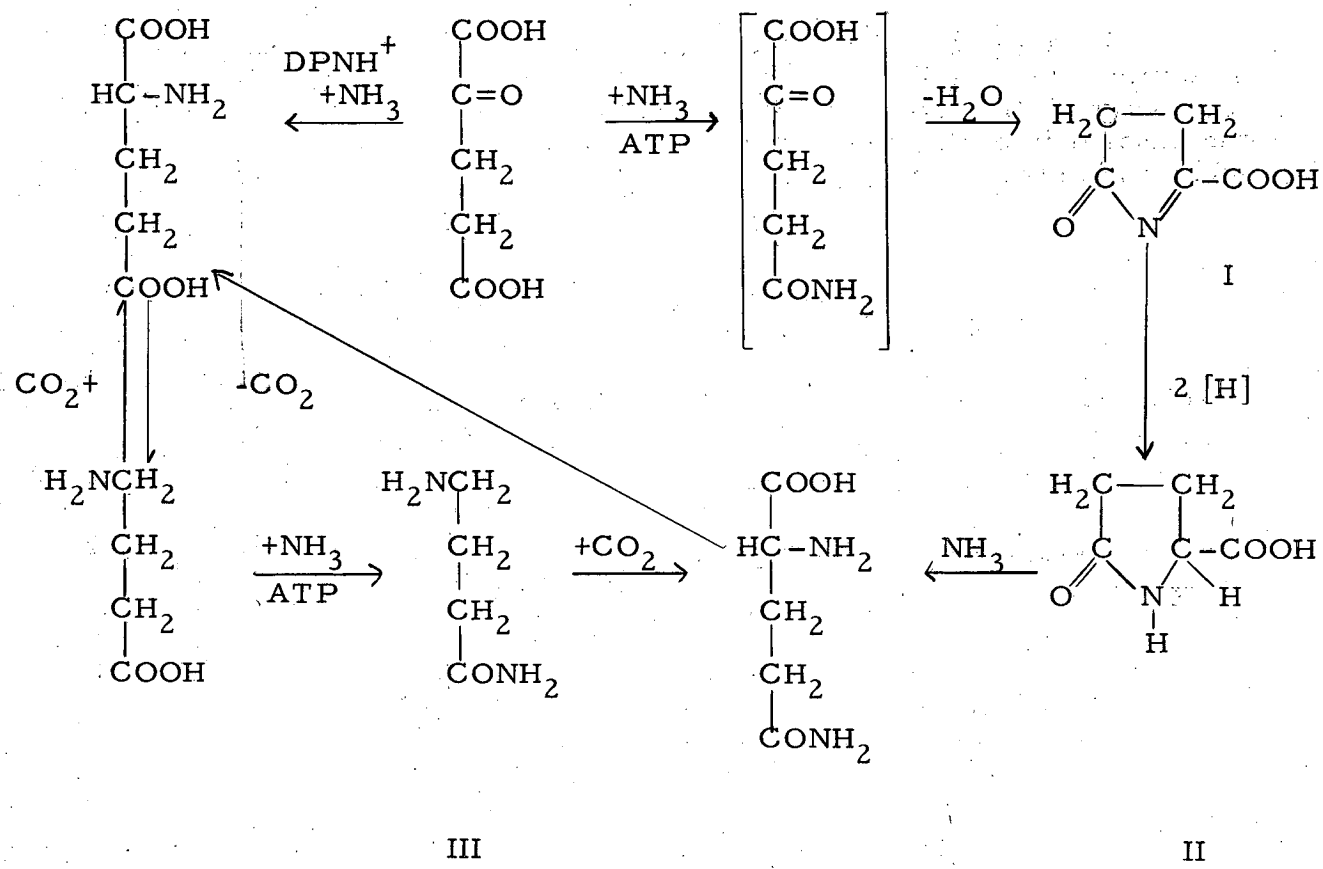


Fig. 2. A second route from glutamine to glutamic acid not involving glutamic acid.

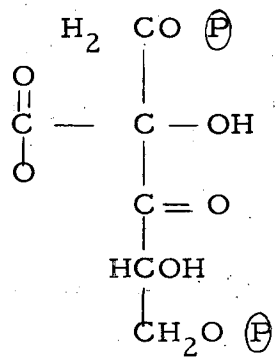
We have thus two alternative routes to labeled glutamine not involving glutamic acid as a direct precursor. The relative importance of these, together with the direct route through glutamic acid, will be determined by the conditions of the experiment. The exact relationships must await a more extensive set of degradations.

THE REACTION OF RIBULOSE-1,5-DIPHOSPHATE WITH CYANIDE

Brian R. Rabin, Duncan F. Shaw, Ning G. Ponn and Joan Mary Anderson

Introduction

In the carboxylation of ribulose-1,5-diphosphate to form 3-phosphoglyceric acid, a β-keto acid is postulated as an intermediate:[1]



It is apparent that if this compound were reduced with a carbonyl specific reducing agent (KBH_4), a hydroxy acid should be formed. The dephosphorylated compound would be a polyhydroxy acid related to hamamelonic acid or its isomer. On this basis experiments were carried out on the carboxydismutase system in an attempt to trap the keto acid intermediate.

In the meantime, in this laboratory, Otto Kandler has studied the effect of cyanide on the CO_2 fixation by algae, Chlorella pyrenoidosa.[2] It was found that a new compound appeared in the diphosphate region after chromatography in the usual solvent systems (butanol–propionic acid–water and phenol-water). When this diphosphate area was treated with acid phosphatase, two new spots appeared (in addition to other known sugars). Further characterization by rechromatography and paper electrophoresis showed that these two spots exhibited an acid-lactone behavior. Subsequently, this compound was tentatively identified as hamamelonic acid.[3,4]

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

[2] O. Kandler, in Chemistry Division Quarterly Report, UCRL-3710, March 1957, p. 9.

[3] O. Kandler, (UCRL) personal communication.

[4] J.M. Anderson and D.F. Shaw, in this Chemistry Div. Quarterly Report, p.63.

In order to make further studies, carrier amounts of this compound must be made available. A search of the leterature has shown that hamamelonic acid can be prepared by reaction of ribulose with HCN.[5] (A small amount of an authentic sample of hamamelonic acid was kindly supplied by O. Schmidt). Also it is desirable to have the diphosphate. It is the purpose of this report to describe the preparation of the diphosphate.

Experimental Procedures

Reaction of RuDP (ribulose-1,5-diphosphate) with Radioactive Cyanide

Reactions were carried out at calculated pH's of about 6 and 11. For the reactions at pH 6, 0.1 M HCl was used for washing, while for the reaction at pH 11, water was used. The monobarium salt of RuDP (12 mg) was triturated with water (1 ml) and Na_2SO_4 (40 μM). The residue of $BaSO_4$ was removed by centrifugation. The residue from the reaction at pH 6 was washed with 0.1 M HCl (200 μl); the residue from the reaction at pH 11 was washed with water. The combined supernatant and washings were treated with 1.3 mg of $KC^{14}N$ (20 μmole--1μC/μM) and allowed to react at room temperature for 16 hours.

Reaction of RuDP with KCN

The monobarium salt of RuDP (117 mg) was triturated with water (10 ml) containing K_2SO_4(75 mg). The insoluble material was removed by centrifugation and KCN (900 μl of 0.24 M) was added. After 18 hr at room temperature the solution was centrifuged and the small amount of residue was discarded. The supernatant was cooled in ice, and barium acetate (600 μl of 1 M) was added. The copious precipitate was centrifuged off, washed with 5-ml portions of 80% and 90% ethanol, and dried *in vacuo* over P_2O_5; yield, 120 mg. A further 15 mg of the product could be recovered by the addition of ethanol (2 ml) to the supernatant. The material was contaminated with $BaSO_4$, which was removed by trituration with HCl (10 ml of 0.1 M) and by centrifugation. The residue was washed with water (5 ml); it weighed 42 mg and consisted mainly of $BaSO_4$.

The solution was adjusted to pH 7.1 and then cooled in an ice bath and the residue of what we shall call Product A was centrifuged off, washed with 50% ethanol (7.5 ml) and 90% ethanol, and dried *in vacuo* over P_2O_5; yield, 26 mg. A further crop was obtained by adding equal volumes of ethanol to the supernatant. This was centrifuged, washed with 50% ethanol (4 ml) and 90% ethanol, and dried *in vacuo* over P_2O_5; yield, 22 mg of Product B.

Both A and B were cochromatographed with the products of the reaction of $KC^{14}N$ on RuDP. The solvent system of Cowgill, as modified by Trudinger, was used (see below). Coincidence of radioactivity and the phosphomolybdate color were obtained.

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

Identification and characterization

Both the products of the reactions of RuDP with KCN and $KC^{14}N$ were chromatographed in the following solvent systems: (a) Two dimensions in the usual solvent systems, butanol–propionic acid–water and phenol-water,[6] (b) butanol-propionic acid-water (48 hr), (c) propanol-ammonia-water,[7] and (d) ethanol–formic acid–aqueous molybdate (Cowgill).[8] In the first three systems, Whatman No. 4 papers washed with oxalic acid–water were used, while in the last system, Whatman No. 1 papers washed with HCl–ethylenediamine tetraacetate–water were used.

Dephosphorylation was done by enzymic treatment and acid hydrolysis. The acid phosphatase used was a purified fraction obtained from Polidase-S.[9] The conditions for hydrolysis: 25 to 100 μg per spot; pH 5.) (0.2 M acetate containing Mg^{++}); 16 hr at room temperature. Acid treatment consisted of heating the product with 1 M HCl for 16 hr at 120°C in a sealed tube (heating with 0.1 M HCl at 103°C for 12 hours did not seem to dephosphorylate the compound).

Paper electrophoresis was performed at pH 9.1 in NH_3-NH_4^+ buffer (acetate), 0.1 M, at room temperature for 3 hr. A horizontal strip apparatus was used.[10] A potential of 600 volts was applied; the current varied from 10 to 15 ma.

Phosphate esters were detected on paper by spraying with molybdate reagent,[7] while the acid and lactone were detected with an ammoniacal silver spray.

Results

When RuDP is allowed to react with cyanide, at either pH 6 or 11, the reaction proceeds to nearly 100%, allowing for impurities present in the original RuDP sample. The extent of the reaction was followed by taking a known aliquot of the $KC^{14}N$ addition product, acidifying with acetic acid, and counting it.

Chromatography in two dimensions in the usual solvent systems gave 87% of the radioactivity in the diphosphate region. When the product was chromatographed in the butanol–propionic acid–water system for 48 hr and compared with RuDP, no apparent separation was achieved. However, in the ethanol–formic acid–molybdate system and the propanol-ammonia-water system a separation of RuDP from the cyanide addition product was effected. In the propanol-ammonia-water solvent system, the RuDP appeared to be unstable and therefore to be destroyed on chromatography. At least two spots were obtained when the addition product was chromatographed in the last two solvent systems.

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[6] A. A. Benson, Moderne Methoden der Pflanzenanalyse, K. Paech and M. V. Tracey, Eds. (Springer-Verlag, Berlin, 1955), II, p. 113.

[7] C. S. Hanes and F. A. Isherwood, Nature 164, 1107 (1949).

[8] P. A. Trudinger, Biochem. J. 64, 274 (1956).

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

[10] A. B. Foster, Chem. Ind. (London) 1050 (1952).

On dephosphorylation of the radioactive diphosphate area with acid phophatase and two-dimensional chromatography of the reaction mixture in the usual solvent, many spots were obtained. Among the major spots were the original diphosphate, and what appeared to be an acid spot and a lactone spot.

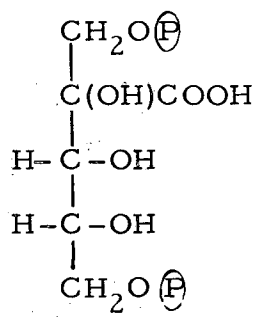
Acid treatment of the radioactive cyanide-RuDP mixture yielded, on chromatography in two dimensions, a spot that was in close coincidence with that of authentic hamamelonic acid (lactone). The fact that the spot did not show exact coincidence is indicative of the presence of the two isomers, namely, hamamelonic acid and its epimer.

Paper electrophoresis of the original reaction mixture gave rise to at least three radioactive spots, of which the major one had the greatest mobility towards the anode. It should be noted here that RuDP has about the same mobility and hence is indistinguishable from the reaction product. Furthermore, 3-phosphoglyceric acid (PGA) has a greater mobility than either of these compounds; although at this pH the number of charges in PGA is 3 while the number of charges in RuDP is 4 and in the reaction product is 5. It appears that a large factor in the mobility is the charge-to-weight ratio, when the major spot and a minor spot were phosphatased, an acid and a lactone were obtained that have R_f's similar to those of hamamelonic acid and its lactone when chromatographed two-dimensionally in the usual solvent systems.

Conclusion

It has been shown, by its chromatographic properties in several different solvent systems, that the cyanide addition product of RuDP is a diphosphate. That this diphosphate is not identical to RuDP is evident by chromatography in the ethanol-formic acid-molybdate and propanol-ammonia-water solvent systems, and furthermore by the marked stability of the phosphate ester towards acid and alkaline hydrolysis. RuDP, on the other hand, is exceedingly unstable to acid at elevated temperatures and to alkali at room temperature.[11]

The observations that acid hydrolysis of the $C^{14}N^-$ addition product gave a spot which cochromatographed with authentic hamamelonic acid lactone and that the action of acid phosphatase also gave a similar spot suggest that the end product contains a carboxyl group on the 2-C position and not a cyanohydrin. Since RuDP is a 1,5-diphosphate of a 2-ketopentose, then the structure of the reaction can be tentatively assigned:



[11] Horecker, Hurwitz, and Weissbach, J. Biol. Chem. 218, 785 (1956).
Calvin

In summary, the significance of this compound in photosynthesis is its similarity to (a) the reduction product of the β-keto acid, and (b) the new compound obtained by O. Kandler in cyanide-inhibited Chlorella. In the latter case, it has been demonstrated that the above RuDP-CN^- product and the product obtained in vivo are one and the same.[4]

RESPONSE OF CHLORELLA TO A DEUTERIUM ENVIRONMENT

V. Moses and Osmund Holm-Hansen

The work reported in the present paper is a continuation of investigations described previously.[1,2]

Intracellular Deuterium Concentrations in Unadapted and Adapted Cells Grown in the Presence of D_2O

(a) Unadapted Cells

A 25-ml amount of cell suspension containing a packed cell volume of 0.125 ml of unadapted cells was inoculated into 975 ml of growth medium, producing a final concentration in the medium of about 23.5% D_2O. The cells were grown in the presence of air + 4% (v/v) D_2O for 8 days. The cells were harvested; the volume of the packed cells after growth was 9 ml; there was thus a 72-fold increase in cell material during growth.

The deuterium content of the water of the medium, of the intracellular water obtained by lyophilization of the packed cells, and of the water produced by combustion of the dried cells was measured. The D_2O concentrations of these three samples were 21%, 24%, and 13%, respectively, confirming the findings of Weinberger and Porter that unadapted cells select protons from the growth medium in preference to deuterons.[3]

(b) Adapted Cells

A similar experiment has been performed with adapted cells.[2] After algae previously adapted to grow in the presence of 60% D_2O had been grown in 1000 ml of medium containing this level of D_2O, the medium contained 63.7% D_2O, the intracellular water 63.4% D_2O, and the water obtained by combustion of the dried cells had a D_2O content of 69.8%. The phenomenon of adaptation thus involved a shift from the preferential absorption of H from the medium by unadapted cells to a preferential absorption of D by cells growing in the presence of D_2O. This may imply a considerable alteration of the proton-deuteron composition of the genetic and enzymic constituents of the adapted cells.

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[1] Holm-Hansen, Moses, and Yarberry, in Chemistry Division Quarterly Report, UCRL-3629, Jan. 1957, p. 31.

[2] V. Moses and O. Holm-Hansen, in Chemistry Division Quarterly Report, UCRL-3710, March 1957, p. 22.

[3] D. Weinberger and J. W. Porter, Arch. Biochem. Biophys. 50, 160 (1954).

Calvin

Effect on Photosynthesis of Increasing Deuterium Content

Unadapted cells were suspended in distilled water containing 0%, 33%, 66%, and 99.5% D_2O, at the same cellular concentration in each suspension. Each suspension was supplied with equal quantities of $NaHC^{14}O_3$, and photosynthesis was allowed to proceed for 3 minutes. The total quantities of C^{14} fixed, the quantities present in the hot ethanol extracts, and the percentages of the total ethanol-soluble activities present in a number of intermediary metabolites are given in Table XII(A).

A similar experiment was performed with cells adapted to and grown in 60% D_2O. The results are shown in Table XII(B). A final experiment was performed in which cells adapted to grow in 60% D_2O were grown in four D_2O concentrations, 0%, 20%, 40%, and 60%, and were resuspended in distilled water containing these concentrations of D_2O. After 3-min periods of photosynthesis the cell extracts were analyzed as above; the activities in the various fractions are presented in Table XII(C).

The effects of deuterium oxide on the metabolism of Chlorella can be separated to some extent into those changes due to the presence of D_2O in the environment with little or no incorporation into the larger molecules of the cells, and those associated with adaptation of the cells with the consequent utilization of relatively large amounts of deuterium for general synthetic purposes. The lowered rates of the incorporation of $C^{14}O_2$ by both adapted and unadapted cells shows evidence that this effect is due largely to a deuterium environment, at least up to the concentration of D_2O to which adapted cells have been adapted. Unadapted cells photosynthesizing in 66% D_2O fix only 0.2% of the C^{14} taken up into the ethanol-insoluble fraction. Adapted cells grown in 66% D_2O and suspended subsequently in 66% D_2O fixed 4% in this fraction, while in another experiment cells grown and suspended in 60% D_2O converted 11% of the C^{14} utilized into insoluble material.

The presence of a much larger pool of radioactive phosphoglyceric acid in all adapted cells than in unadapted organisms indicates that incorporation of deuterium into enzymic material during adaptation may impair the subsequent reduction of this substance. The rise in the ratio of radiocarbon found in ribulose diphosphate and in phosphoglyceric acid appears to be affected more by the D_2O concentration present during the photosynthesis experiment than by that during growth, suggesting that D_2O may interfere with an early stage in CO_2 absorption. However, except at the highest D_2O concentrations, the sums of the percentages of the total activities in ribulose diphosphate and phosphoglyceric acid are roughly equal in all cases. The inhibitions of phosphoglyceric acid reduction and of CO_2 fixation lead to some drop in the activities in the sugar phosphates as a whole, with fluctuations in individual species, which probably may be ascribed to uncontrolled changes in the experimental environment. The incapacity of the adapted cells for rapid protein synthesis leads to an accumulation of some amino and organic acids. Adapted cells fix appreciable amounts of C^{14} into citrulline; although activity appears in citrulline in unadapted cells placed in high concentrations of D_2O. Larger amounts of citrulline are present at all D_2O concentrations in cells grown in 60% D_2O. The growth of cells adapted to 60% D_2O for one subculture in low concentrations of D_2O leaves a capacity for incorporating $C^{14}O_2$ into citrulline greater than that of unadapted cells.

Table XII

Percentages of the total C^{14} in a number of known substances that are found in the ethanol-soluble fraction.

A, unadapted cells suspended in the D_2O concentrations shown. B, cells adapted to and grown in 60% D_2O, and suspended in the D_2O concentrations shown. C, cells adapted to grow in 60% D_2O, grown in the D_2O concentrations shown, and suspended in the same concentrations of D_2O for study of photosynthesis.

	A				B				C			
% D_2O in growth medium	0	0	0	0	60	60	60	60	0	20	40	60
% D_2O in suspension medium	0	33	66	99.5	0	33	66	99.5	0	20	40	60
Ribulose monophosphate	0.59	0.64	0.83	1.2	0.29	0.17	0.27	0.0	0.22	0.19	0.30	0.0
Fructose monophosphate	9.2	9.0	9.1	9.3	11.2	5.2	6.7	8.6	9.5	12.3	11.5	13.2
Glucose monophosphate and Sedoheptulose monophosphate	27.8	20.4	16.4	14.9	19.7	14.3	12.8	11.4	31.5	28.8	23.0	22.3
Ribulose diphosphate	10.3	16.7	21.0	23.3	1.9	1.8	3.0	3.1	0.74	2.8	2.9	2.0
Fructose diphosphate	2.3	1.0	0.68	0.76	2.4	0.7	1.0	1.5	2.1	2.0	0.79	2.2
Glucose diphosphate	0.79	0.66	0.54	0.49	0.44	0.23	0.49	2.4	0.87	0.88	0.97	0.45
Uridine diphosphoglucose	3.9	3.2	3.6	3.1	3.8	1.4	1.0	0.56	3.2	3.3	3.5	1.0
Phosphoglyceric acid	9.9	7.9	10.7	11.1	18.1	12.2	17.4	19.4	26.8	22.5	25.6	11.1
Phosphoglycollic acid	1.1	7.3	3.1	9.5	0.64	0.86	0.56	0.92	1.7	1.2	1.4	1.2
Phosphopyruvic acid	0.0	0.0	0.0	0.0	1.7	0.57	0.0	0.0	0.30	0.74	0.73	0.23
2-Carboxy, 3-ketopentose-1:5-diphosphate	0.82	0.67	0.68	0.40	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Fumarate	0.66	0.82	0.84	0.93	2.7	2.2	2.1	2.4	0.57	0.78	1.5	3.3
Malate	3.9	5.8	5.6	7.1	16.5	11.3	14.7	15.6	2.7	4.1	9.7	19.3
Citrate	0.05	0.04	0.25	0.87	1.1	2.6	2.0	1.6	0.0	0.0	0.14	0.97
Aspartate	2.9	1.6	1.5	1.2	4.5	10.4	9.2	11.7	2.8	5.1	4.9	5.0
Alanine	5.5	5.5	5.9	4.5	3.1	13.5	13.7	9.7	8.8	5.0	2.7	3.4
Citrulline	0.0	0.0	0.12	0.73	2.1	2.7	1.8	1.9	0.0	0.16	0.27	2.4
Glycine	0.78	0.45	0.0	0.0	0.10	0.43	0.20	0.56	0.30	0.24	0.36	0.0
Serine	3.0	2.1	1.6	1.0	1.9	3.4	2.0	2.3	1.7	2.4	2.5	2.7
Glutamic acid	0.0	0.08	1.2	1.2	1.5	6.1	4.9	3.1	0.0	0.18	0.42	2.0
Sucrose	10.4	9.6	11.0	2.9	0.0	0.25	0.18	0.0	2.1	2.6	2.4	0.0
Unidentified 1	2.0	2.2	2.5	2.0	1.6	1.3	1.5	1.2	3.0	2.6	2.6	1.1
Unidentified 2	0.39	0.42	0.56	0.45	0.0	0.07	0.21	0.69	0.19	0.21	0.08	0.90
Unidentified 3	0.25	1.2	0.81	2.0	0.30	1.4	1.3	0.0	0.0	0.0	0.0	0.52
Total recovered	96.53	97.28	98.51	98.93	95.57	93.08	97.01	98.63	99.09	98.58	98.26	95.27
Total in phosphates	66.70	67.47	66.63	74.05	60.17	37.43	43.22	47.88	76.93	75.21	70.69	53.68
Total in organic acids	4.61	6.66	6.69	8.90	20.30	16.10	18.80	19.60	3.27	4.88	11.34	23.57
Total in amino acids	12.18	9.73	10.32	8.63	13.20	36.53	31.80	29.26	13.60	13.08	11.15	15.50
Total $C^{14}O_2$ fixed per 100 µl packed cells (counts/min x 10^{-5})	54.23	37.74	40.06	27.91	22.94	10.70	5.15	1.87	3.52	4.52	5.74	5.53
% in EtOH-soluble fraction	83	89	99.8	99.5	90	93	96	99	80	77	82	89

Unadapted cells show an abnormally low incorporation of CO_2 into sucrose when placed in 99.5% D_2O, but adapted cells exhibit this effect even when placed in H_2O. A possible explanation of this is the need of adapted cells to utilize the reduced amounts of fixed CO_2 available for synthesis, with a resultant fall in the "luxury" production of sucrose.

GLUCOSE AND CARBON DIOXIDE INCORPORATION IN A FUNGUS

V. Moses

Studies with the phycomycete mold Zygorrhynchus moelleri have shown that there is a very high endogenous respiration on removal of an organism from the growth medium, which masks the effect of added respirable substrate. Starvation of the fungus in phosphate buffer for 24 hours markedly reduces the rate of endogenous respiration, but there is a lag period of 2 to 3 hours after subsequent addition of glucose before the rates of gas exchange become constant at their maximal values.[1] Analysis of this phenomenon of the lag period has indicated that three factors are involved:

(a) Enzyme adaptation. If the cells are starved only of carbohydrate and not of nitrogen a reduction in the final rate of gas exchange is observed in the presence of inhibitors of enzyme synthesis (dinitrophenol, azide, and cyanide). When the cells are starved of nitrogen as well as of carbohydrate (by being suspended in inorganic phosphate buffer) a stimulation of the respiration rate follows the addition of small amounts of ammonia together with glucose--a stimulation greatly in excess of that to be expected if the ammonia nitrogen is used for general cell growth. This stimulation of the respiration by ammonia continues long after the ammonia has disappeared from the medium, and is very sensitive to those inhibitors mentioned above.[1]

(b) Permeability. There is some evidence that a fairly small part of the lag period may be due to a delay before the internal glucose concentration builds up to a level high enough to saturate the enzyme systems involved in its metabolism.[2]

(c) Reduction during starvation of one or more metabolic pools of intermediates connected with glucose utilization. This is probably the major factor accounting for the lag period; the latter can be reduced very markedly by supplying the starved cells with small amounts of acetate together with glucose.[3]

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[1] V. Moses, Biochem. J. 57, 547-556 (1954).

[2] V. Moses, J. Exptl. Botany 6, 222-234 (1955).

[3] V. Moses, Ann. Botany (London) 19, 211-223 (1955).

Work with labeled substrates showed that the tricarboxylic acid cycle is a major pathway for both oxidative and synthetic processes.[4] Evidence has also been obtained that some role in glucose utilization is played by the hexose monophosphate shunt pathway.

In a program of kinetic investigations of the short-period metabolism of labeled glucose by starved and unstarved hyphal preparations of the fungus, preliminary experiments have been performed to obtain some idea of the rate at which the metabolic reactions are likely to be carried out, and to identify as many products of glucose metabolism in as short a period as possible. Both starved and unstarved cells have been supplied with glucose-U-C^{14} for periods of 5, 20, and 45 sec, 3 min, and 30 min. After rapid filtration (1 to 2 sec) of the suspensions to remove the bulk of the unused glucose, the cells were killed with boiling 80% ethanol, extracted with this and with boiling 20% ethanol, and chromatographed by use of the procedures standard for photosynthesis studies. Table XIII shows the sequence of incorporation of C^{14} from glucose into unstarved cells, together with a rough estimate of the activities in each compound.

A similar experiment has been performed with starved cells. The following compounds have been found to incorporate C^{14} from glucose-U-C^{14} in 3 min: fructose diphosphate; uridine triphosphate; uridine diphosphoribose; uridine diphosphoglucose; a nucleotide mixture (containing uridine, guanosine, and inosine diphosphates and an unknown component); glucose, ribose, fructose, and sedoheptulose monophophates; phosphoglyceric acid; phosphoglycolic acid; alanine; glutamic, aspartic, malic, and citric acids; maltose; probably glycerol phosphate and guanosine monophosphate; and possibly threonine. After 30 minutes the following additional compounds were active; some unidentified substances in the phosphate area; histidine; tyrosine; glutamine; succinic and fumaric acids; and probably citrulline, together with some weakly active unknown substances.

The most prominent difference between starved and unstarved cells was the very high activity in starved cells found in maltose; after 30 minutes this was the single most active compound in starved cells.

Further work of a preliminary nature has also been performed to investigate the pathway of carbon dioxide fixation in unstarved cells of this organism. It is of considerable interest in this case that whereas many of the tricarboxylic-acid-cycle organic and amino acids are highly active, activity is present in many of the sugar phosphates also--except, apparently, fructose diphosphate. Fructose and glucose monophosphates, however, incorporate activity from $C^{14}O_2$, as does phosphogluconic acid. It would therefore appear that activity does not enter the hexoses from the Krebs cycle by a reversal of glycolysis, for no activity was found in fructose diphosphate. This compound is known to be present, as it has been found labeled in cells supplied with labeled glucose (see above). However, even when $C^{14}O_2$ is supplied to cells metabolizing unlabeled glucose simultaneously, there is still no activity in fructose diphosphate. The presence of labeled hexose monophosphates and phosphogluconic acid, although the latter is in very small amounts, suggests that the incorporation of carbon dioxide may

[4] V. Moses, J. Gen. Microbiol. 16, 534-549 (1957).

proceed to some extent by the reversal of the decarboxylation of phosphogluconic acid to pentose phosphate.

Ribulose diphosphate, which becomes labeled during the photosynthetic assimilation of $C^{14}O_2$, was not observed during experiments on $C^{14}O_2$ fixation by this mold, indicating that the phtosynthetic carbon cycle is not operating in this nonphotosynthesizing organism.

Table XIII

Metabolism of radioactive glucose by unstarved cells
(counts/min in standard aliquots after feeding glucose-U-C^{14})

Compound	Time during which glucose-C^{14} is present				
	7.2 sec	21 sec	0.47 sec	3 min	30 min
Fructose diphosphate	300	710	805	5385	
Uridine triphosphate	0	0	0	6770	4050
Uridine diphosphoribose	0	0	0	1985	1750
Uridine diphosphoglucose	0	595	1570	9400	4120
Nucleotides (see below)	0	0	0	14400	87715
Sedoheptulose monophosphate (plus some glucose monophosphate)	0	0	0	4500	0
Glucose monophosphate	2480	8075	13725	22855	4450
Ribose monophosphate	0	435	0	1740	0
Fructose monophosphate	1140	3760	7380	12140	2045
Guanosine monophosphate	0	0	0	2550	0
Glycerol phosphate	0	750	300	710	2380
Phosphoglyceric acid	0	1205	2180	6830	1740
Phosphoglycolic acid	0	0	0	2110	650
Phosphogluconic acid	200	520	804	1652	316
Fumaric acid				*	*
Succinic acid					?
Malic acid		*	*	*	*
Citric acid				*	*
Glutamic acid				*	*
Alanine		*	*	*	*
Glutamine		*	*	*	*
Aspartic acid			*	*	*
Valine			*	*	*
Tyrosine				*	*
Histidine					*
Proline					*
Glycolic acid					*
Maltose				*	*

*Present but not counted

Nucleotide mixture, on phosphatasing, contained uridine, inosinic acid, and possibly guanosine and glucose cyclic phosphate. The unphosphatased mixtures thus appeared to contain uridine, guanosine, and inosine diphosphates and an unknown component accounting for the glucose cyclic phosphate.

TRACER STUDIES WITH OXYGEN-18 IN CHLORELLA PHOTOSYNTHESIS

Ulrich Blass

Earlier this year Ingrid Fogelstrøm-Fineman et al. developed a method for the activation analysis of O^{18}-containing metabolites.[1] The report presented here describes some applications of this procedure in photosynthesis with Chlorella pyrenoidosa.

The first experiments were designed to compare the influence of dark and light exposure (1 min photosynthesis) on the formation of metabolites containing O^{18}. Figures 3, 4, and 5 show autoradiographs of paper chromatograms of algae extracts after transfer to tantalum sheets and bombardment with protons. One strip in each figure belongs to the algae exposed to the light, the other to those kept in the dark. The extracts represented in the three chromatograms differ in the procedure of extraction and the solvent for chromatography as specified with each figure. (In Fig. 5 the x-ray film did not touch the metal sheets as closely as in other experiments.) In addition, Figs. 4 and 5 show parallel chromatograms in the indicated solvents of $C^{14}O_2$-exposed algae (1-min photosynthesis). There are definite but only minor differences in radioactivity obtained upon activation of the O^{18} from algae grown in the light and in the dark. Markedly different radioactive spots were obtained, however, from chromatograms of Chlorella grown with $C^{14}O_2$ and Chlorella grown with H_2O^{18}. This clearly indicates different pathways for the incorporation of $C^{14}O_2$ and H_2O^{18} into algae.

The lack of a major difference in O^{18}-induced radioactivity of algae grown in the light and in the dark could have had several reasons, such as too short a light period, too low isotope content, incomplete transfer of organic materials from the paper to the metal, etc. The following series of experiments was therefore designed to check some of these points. Figures 6 and 7 show the autoradiographs of the paper chromatograms transferred to metal and bombarded with protons from algae of which one half had been treated in normal water, and the other half in O^{18}-water (~15% H_2O^{18}). As before, the figures show the parallel chromatograms of $C^{14}O_2$-treated algae. Again, there are only minor differences in radioactivity of the samples grown in H_2O and H_2O^{18}. We therefore extended the photosynthesis periods. Figures 8 and 9 show autoradiographs after 30 minutes' photosynthesis with normal water and two different samples of O^{18}-water (~15% and 30% H_2O^{18}).* In these two pictures some areas of higher radioactivity are indicated.

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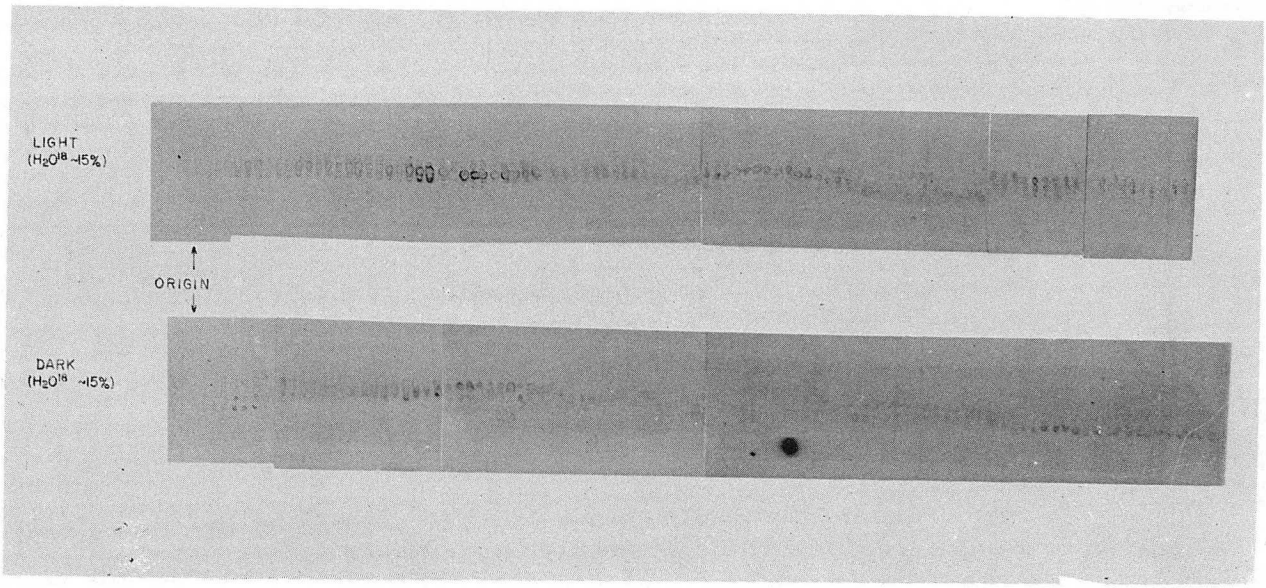
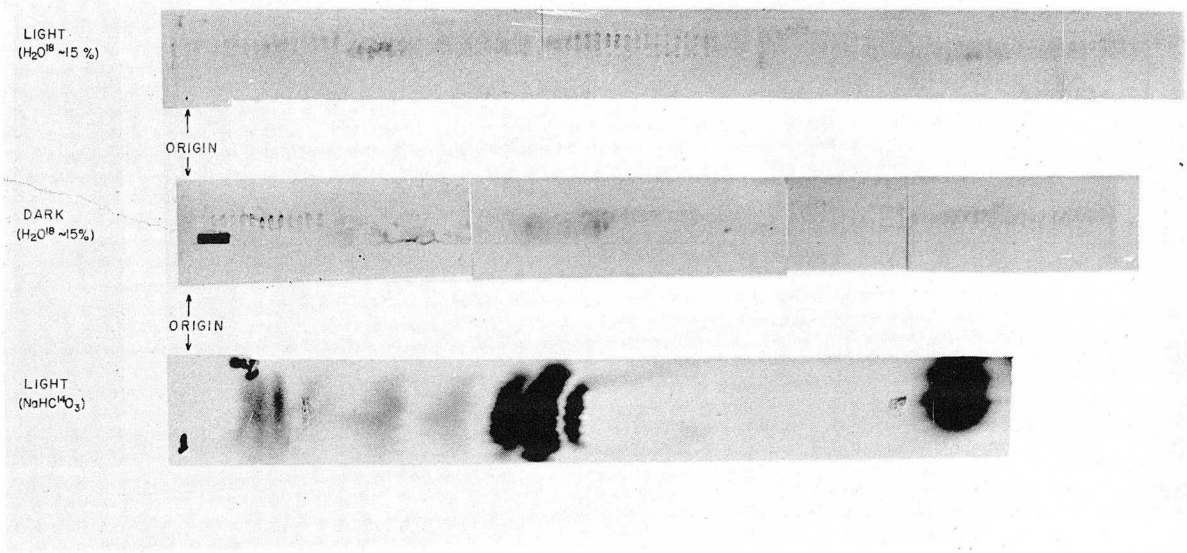
[1] Fogelstrøm-Fineman, Holm-Hansen, Tolbert, and Calvin, Intl. J. Appl. Radiation and Isotopes, in press; UCRL-3790, May 20, 1957.

*Mr. Jeff Davis determined the O^{17} content in the H_2O^{18} samples using the NMR method. His analysis and the original analysis on the sample label were used to estimate the O^{18} content.



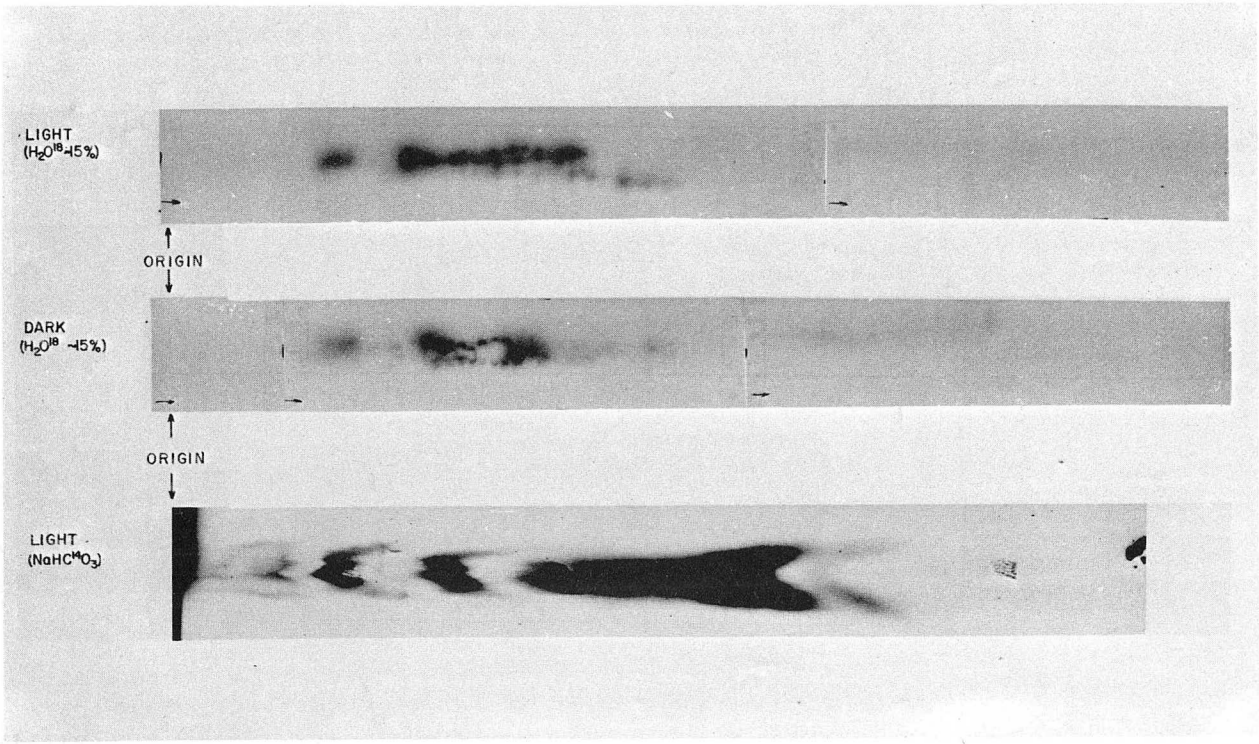
ZN-1800

Fig. 3. Autoradiographs of paper chromatograms from algae extracts after transfer to metal sheets and bombardment with protons.
Chlorella, 1 min photosynthesis in H_2O^{18}
Extraction: 2 x 1 ml boiling methanol, 1 x 1 ml hot water
Solvent: Butanol – propionic acid – water



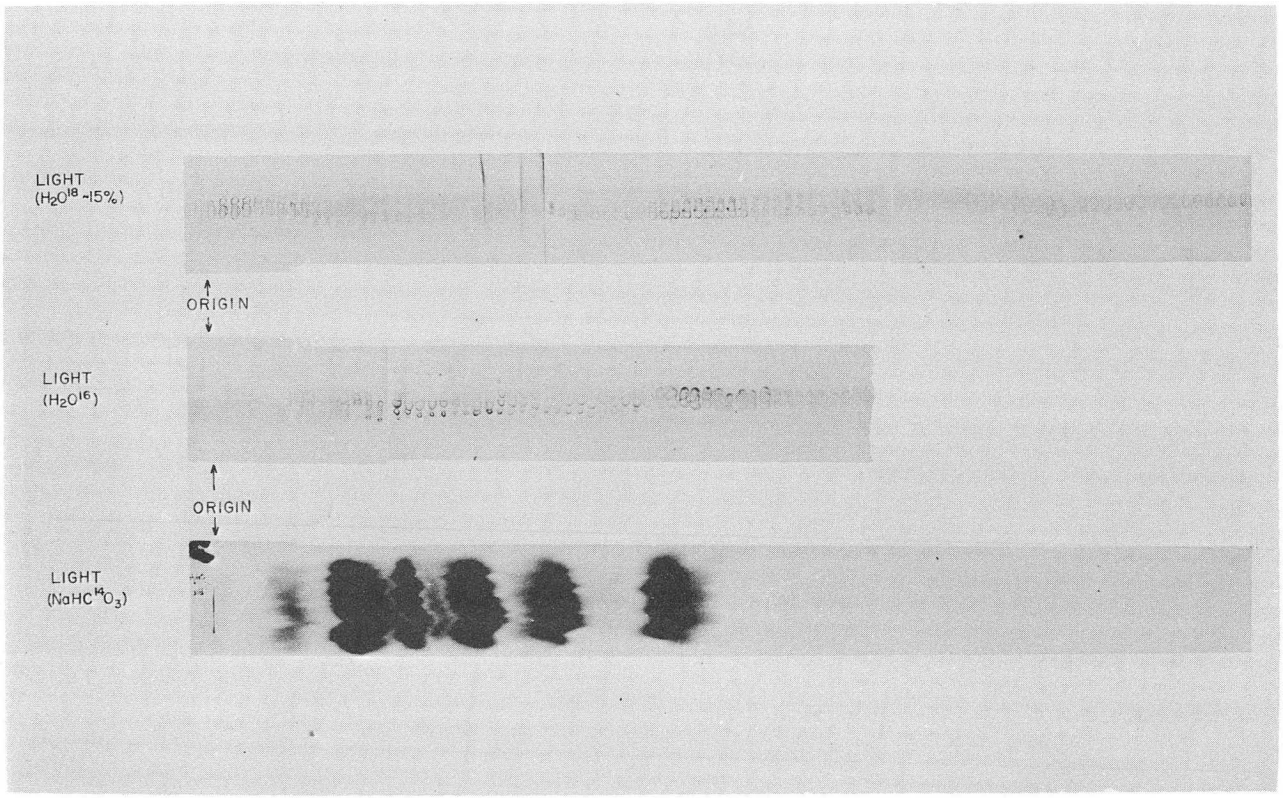
ZN-1801

Fig. 4. Autoradiographs of paper chromatograms from algae extracts after transfer to metal sheets and bombardment with protons.
Chlorella, 1 min photosynthesis in H_2O^{18}
Extraction: 2 x 1 ml boiling 80% aqueous ethanol
Solvent: Butanol – propionic acid – water



ZN-1802

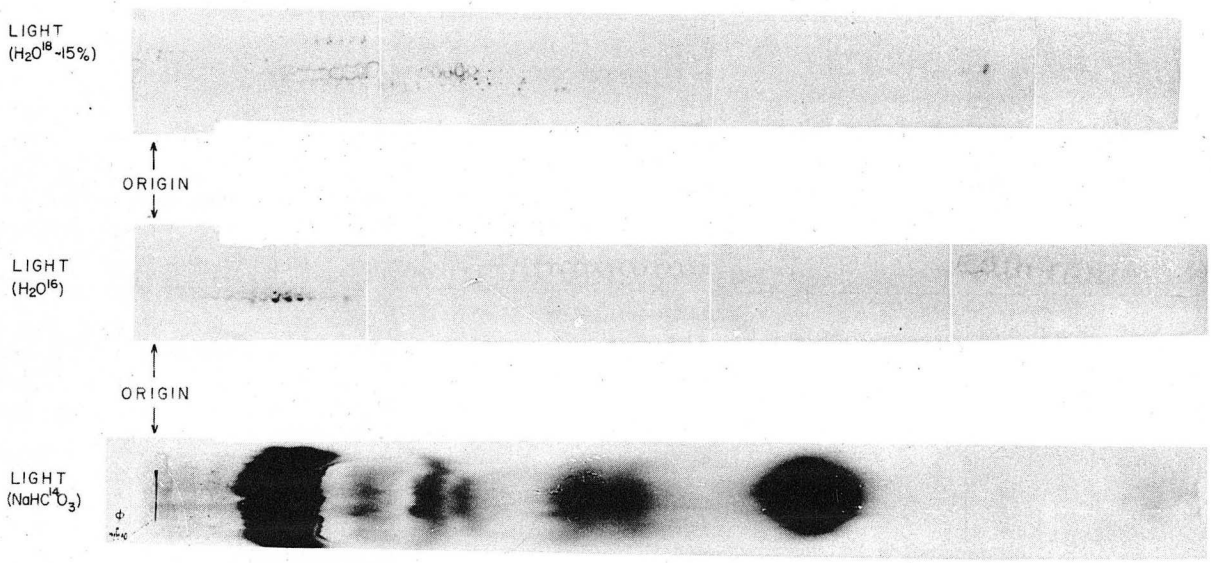
Fig. 5. Autoradiographs of paper chromatograms from algae extracts after transfer to metal sheets and bombardment with protons.
Chlorella, 1 min photosynthesis in H_2O^{18}
Extraction: 2 x 1 ml 80% aqueous ethanol
Solvent: Phenol - water



ZN-1803

Fig. 6. Autoradiographs of paper chromatograms from algae extracts after transfer to metal sheets and bombardment with protons.
Chlorella, 1 min photosynthesis

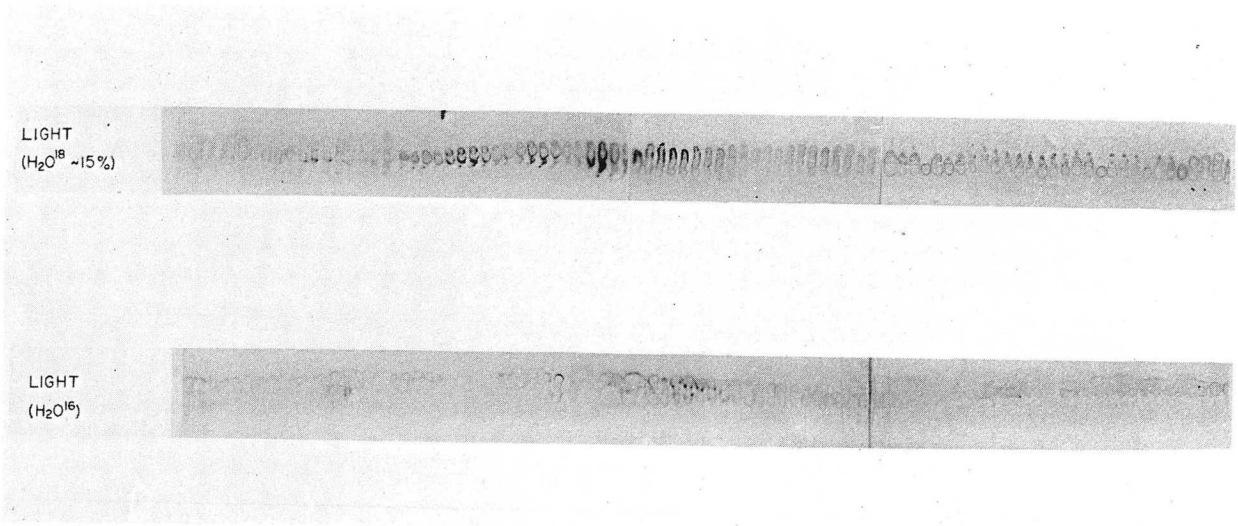
Extraction: Adding boiling ethanol (6 ml)
Solvent: Butanol – propionic acid – water



ZN-1804

Fig. 7. Autoradiographs of paper chromatograms from algae extracts after transfer to metal sheets and bombardment with protons.
Chlorella, 1 min photosynthesis

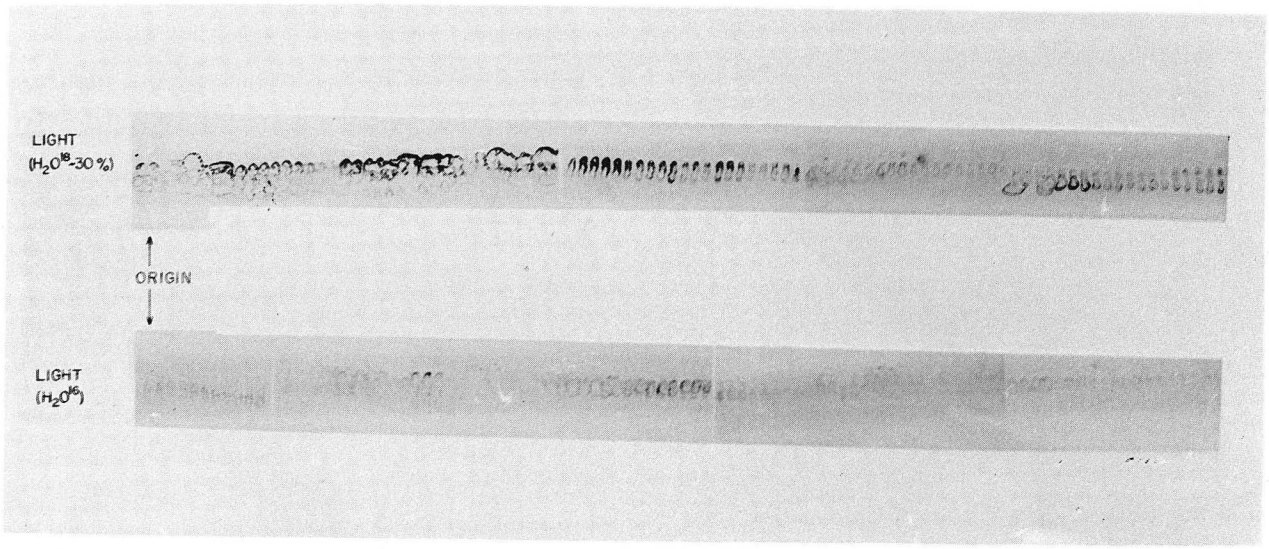
Extraction: 6 ml boiling ethanol
Solvent: Phenol – water



ZN-1805

Fig. 8. Autoradiographs of paper chromatograms from algae extracts after transfer to metal sheets and bombardment with protons.
Chlorella, 30 min' photosynthesis

Extraction: 6 ml boiling ethanol
Solvent: Butanol - propionic acid - water



ZN-1806

Fig. 9. Autoradiographs of paper chromatograms from algae extracts after transfer to metal sheets and bombardment with protons.
Chlorella, 30 min' photosynthesis

Extraction: 6 ml boiling ethanol
Solvent: Butanol – propionic acid – water

In order to get some information about the type of compounds into which the O^{18} is incorporated, we used extracts of algae exposed to $C^{14}O_2$ and some known metabolites. Fructose, glucose, valine, alanine, aspartic acid, and serine were applied parallel to most of the paper chromatograms and then identified by spraying with Tollens' reagent and ninhydrin. These tests indicate, as expected, that the sugar derivatives display a higher O^{18} content than do the amino acids.

Experimental Procedure

The photosynthesis experiments were carried out as follows: 60 microliters of wet-packed cells of Chlorella were suspended in the dark in 3 ml O^{18}-rich water, while a stream of air + 1% CO_2 was bubbled through. One half of the suspension was illuminated for 60 seconds (or 30 minutes, respectively)* with 3000 foot-candles. Both samples were then centrifuged and extracted by the method given with each figure. Extracts from 10 μl of packed algae were then applied to the paper chromatograms. These extracts were transferred to metal sheets as described previously,[1] with the following minor changes in the procedure. The paper chromatograms were eluted with 20% ethanol in water onto tantalum sheets and these sheets heated with heating blades (instead of a hot bank). The elution apparatus under a glass cover could be raised and lowered by means of a Cenco-Lerner Lab-Jack. The tantalum sheets were then bombarded on a wheel of 12-inch diameter in front of a 4-Mev proton beam from the 60-inch cyclotron. The proton yield was kept between 12 and 18 μah in 2 to 3 hours' bombardment time. DuPont x-ray films were exposed to the radioactive metal sheets at various times and for various intervals after the bombardment, according to the intensity of the radioactivity. The best film exposure was usually achieved by a 12-hour exposure, starting 2 to 3 hours after the bombardment. The previous bombardments[1] needed much shorter film-exposure times, which is partly due to more algae material used and partly to a better yield in the cyclotron; but these reasons can account for only a minor difference in the bombardment yield. It is desirable, therefore, to determine the energy resonance curve for the conversion of O^{18} to F^{18} by means of protons in order to find the maximum yield.

*In the later experiments (Figs. 6 to 9) one half of the sample was suspended in distilled water.

MOLAR RADIOACTIVITY OF SCENEDESMUS PIGMENTS

Ulrich Blass

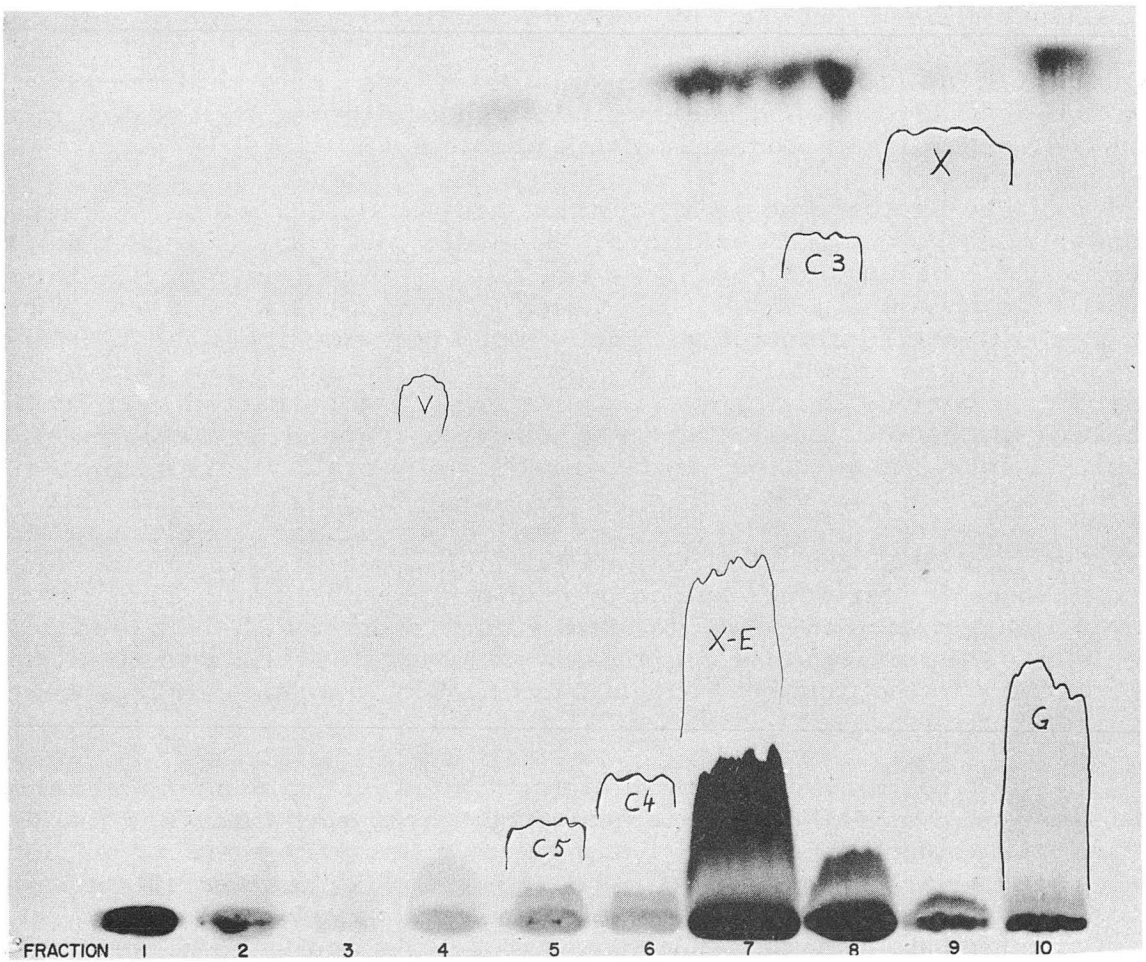
In May 1957 an attempt to determine the molar radioactivity of algae pigments was reported.[1] Scenedesmus algae were allowed to photosynthesize for one hour with $NaHC^{14}O_3$. A methanol extract of the algae was chromatographed on a polyethylene column and the activity of the pigment fractions was determined. Paper chromatograms of these pigment fractions showed that the radioactivity was mostly associated with contaminating colorless compounds. This made it desirable to elute the pigment spots from the paper chromatograms in order to determine the actual concentration and radioactivity. The following report describes further experiments concerning this problem.

The photosynthesis experiment previously described was repeated under slightly changed conditions. Scenedesmus algae (1.2 ml wet-packed cells in 60 ml nutrient solution) were allowed to photosynthesize for 1 hr with 0.5 ml (200 μC) of 0.036 M $NaHCO_3$. The cells were aerated with 4% CO_2 in air and illuminated with 3000 foot-candles of light (one Westinghouse photospot lamp regulated with a Variac). At the end of the 1-hr photosynthesis period, the algae were centrifuged and extracted twice with 10 ml boiling methanol. The extract was chromatographed on a column of 15 g of polyethylene (re-used low-density product of a melt-viscosity molecular weight of 360,000). The colored zones of the methanolic algae extract (80% methanol) were successively eluted with 80% methanol. Solutions of the pigments violaxanthin, C5, C4, xanthophyll epoxide, C3, lutein, and a mixture of the carotenes and chlorophylls were obtained. These solutions were concentrated in vacuo to 20 to 40 ml, diluted with 5% aqueous sodium chloride solution, and the pigments were extracted with 2 to 10 ml benzene (according to the pigment concentration). The benzene extracts were kept frozen until use.

Aliquot portions of these pigment solutions in benzene were applied to Whatman No. 3MM paper. Ascending chromatograms were made in the dark with toluene (Fig. 10). The pigment spots were then cut out and eluted by a variety of solvents. These elutions were carried out as short-time ascending chromatograms in small beakers. When the pigment accumulated in the upper end of the paper, this end was cut out and eluted in 4 to 5 ml of boiling solvent, which was then cooled rapidly to room temperature. The molar radioactivity of the eluted pigments was determined from the concentration obtained from the light-absorption spectrum, and the radioactivity was determined by plating an aliquot portion of the solution. The data obtained for the molar radioactivity of Scenedesmus pigments are summarized in Table XIV.

The green pigments in the last of the elution fractions from the polyethylene column yielded, on paper chromatography, four colored compounds not present in the original chlorophyll a and chlorophyll b. These compounds are artifacts formed during extraction and chromatography

[1] Ulrich Blass, Chemistry Division Quarterly Report, UCRL-3836, June 1957 p 46.



ZN-1807

Fig. 10. Autoradiograph of a paper chromatograph of Scenedesmus pigment fractions obtained after chromatography on a polyethylene column.

V, violaxanthin; X-E, xanthophyll epoxide; X, santhophyll; C5, C4, C3, unidentified monoepoxy carotenoids; G, green pigments.

Table XIV

Molar radioactivity in millicuries per mole of Scenedesmus pigments after one hour of photosynthesis with $NaHC^{14}O_3$ (11.1 curies/mole, max. activity)

Solvent	Pigment									
	Violaxanthin	C5	C4	Xanthophyll epoxide	C3	Lutein	Chlorophyll a	Chlorophyll b	Carotene α	Carotene β
Isopropyl ether	270			7		296				
Isopropyl ether + 10% isopropanol	150 489	436	851	31	310	60	200 178 240 147	 100 56		
Methanol + benzene, 4:1	74	174	13	6	394	78	215 224	120 130		
Petroleum ether									1130 814[a]	622 700[a]

[a]Data from a previous 1-hr photosynthesis experiment with Scenedesmus.

on the polyethylene column by the loss of magnesium and transesterification of the phytol group. An autoradiograph (Fig. 11) of the paper chromatogram showed some radioactivity in all the green spots containing chlorophyll a and its derivatives. Little radioactivity was present in the spots from chlorophyll b. The eluate of the chlorophyll b spot did not have pure chlorophyll b spectrum. It was therefore necessary to chromatograph the green pigments in two dimensions on paper, using petroleum ether (bp 65-100°C) + 3% n-butanol as the second solvent after toluene. The chlorophyll b spot can then be eluted and a solution with a pure chlorophyll b spectrum can be obtained. In order to determine the concentration of chlorophyll, the extinction coefficients listed in Table XV were used.

Table XV

Molar extinction coefficients (ϵ) used for the determination of the chlorophyll concentrations[2]

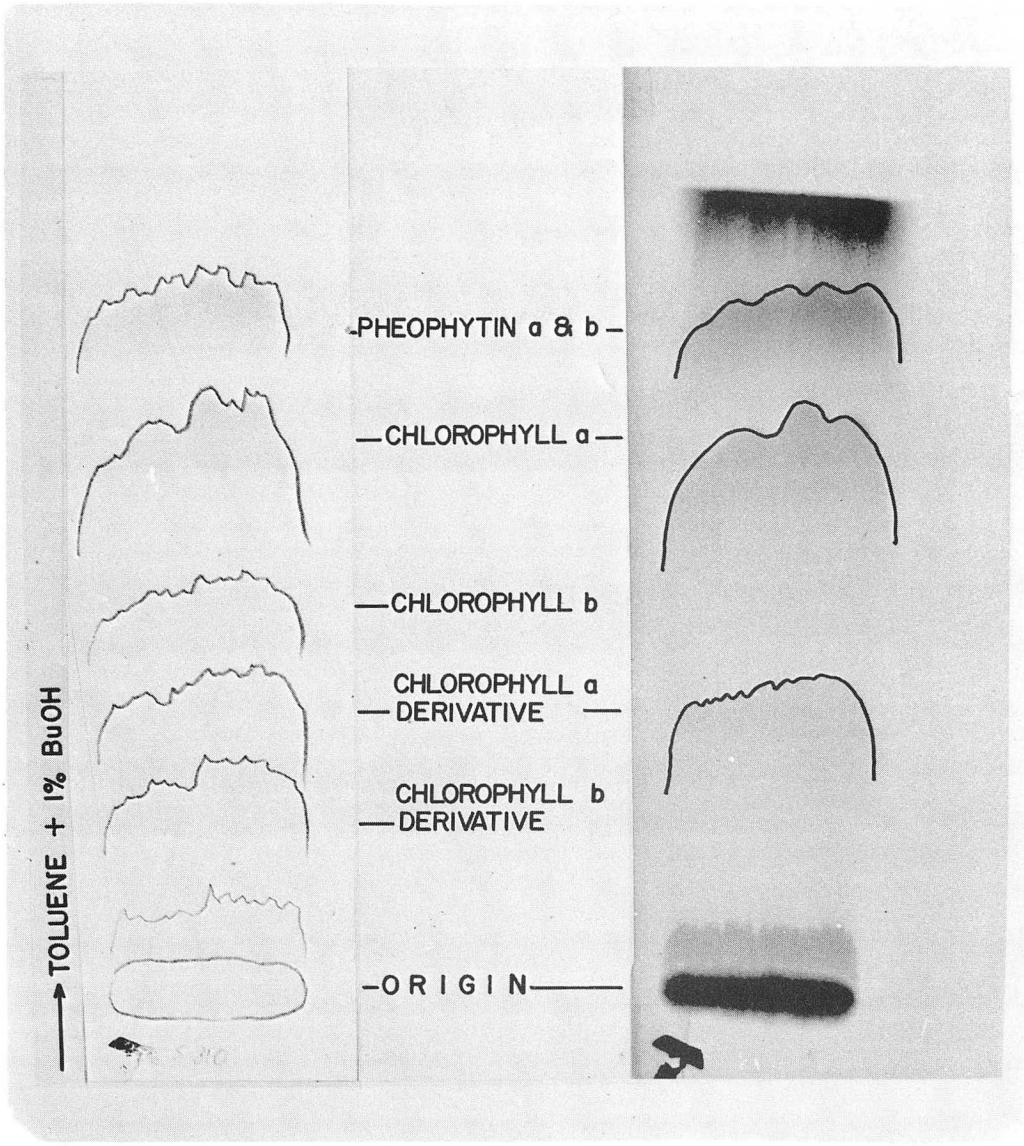
Solvent	Chlorophyll a		Chlorophyll b	
	λ_{max} (mμ)	Extinction coefficient	λ_{max} (mμ)	Extinction coefficient
Isopropyl ether + 10% isopropanol	663	9.1×10^4	645	5.15×10^4
	430	12.0×10^4	455	15.5×10^4
Methanol + benzene, 4:1	665	8.2×10^4	655	4.2×10^4
	432	8.8×10^4	466	12.0×10^4

The two carotenes cannot be separated by paper chromatography. The carotene fraction was therefore rechromatographed twice on a calcium hydroxide column with petroleum ether as a developer. Purified fractions of radioactive α-carotene and β-carotene were obtained. The specific activities of the carotenes were determined , based upon the determination of the concentration from the spectra and radioactivity determinations by direct-plating techniques of suitable aliquots of the solutions. The spectral analyses of the pigment concentration were based on the molar extinction coefficient $\epsilon = 13.5 \times 10^4$ for both carotenoids regardless of the solvent. This introduces a possible error of ± 5%.

Discussion

The data of the molar radioactivity of the pigments presented in Table XIV show very large differences for the same pigment, even though the pigments were obtained from the same extract from one photosynthesis

[2] F. P. Zscheile and C. L. Comar, Bot. Gaz. 102, 463 (1941); D. G. Harris and F. P. Zscheile, op. cit. 104, 515 (1953).



ZN-1808

Fig. 11. Paper chromatogram (left) and autoradiograph (right) of green pigments from Scenedesmus. One hour of photosynthesis with $C^{14}O_2$.

experiment with Scenedesmus (with two specified exceptions). When the main sources of possible errors--(a) fading of the colored spot on the paper, and (b) colorless radioactive impurities--are considered, it is clear that methodical errors tend to yield too high values for specific activity, while other causes of error are of minor order of magnitude. Pigment spots of very low concentration are subject to relatively fast fading, and determinations of their radioactivity are very sensitive to impurities. These considerations apply particularly to the monoepoxy carotenoids C5 and C3, which are present at about 1/20 the concentration of other carotenoids (1/50 the concentration of xanthophyll). Therefore it is believed that the lowest specific activity given for each pigment in Table XIV represents the best data at the present time.

Some further observations that support the same conclusion should be mentioned . In order to determine the radioactivity of the pigment, an aliquot portion (usually 1/10) of the colored eluate was plated. Most of the aliquot portions contained very little radioactivity (e.g., 11 cpm in chlorophyll b). Elution with methanol-benzene, 4:1, led to a marked improvement in the counting accuracy because the pigment could be extracted into the benzene layer after dilution with 5% aqueous sodium chloride. The entire eluted pigment could then be quantitatively transferred to a planchet to determine radioactivity. This method is not without disadvantages, however, since the methanol-benzene solvent requires elution at higher temperatures, which causes some decomposition of the green pigments and thus too high values of specific radioactivity. The main disadvantage of the ether solvents is rapid formation of perioxides which destroy the pigments. The petroleum ether solvent is useful only for the carotenes. The specific radioactivity data for α- and β-carotene may be slightly high owing to a very radioactive preceding zone in the calcium hydroxide chromatograms, which tails slightly into the α-carotene zone even upon rechromatography. The difficulties with radioactive impurities become minor if the original methanolic extract of the plant is hydrolyzed with a suitable amount of potassium hydroxide for 12 to 20 hr at room temperature. Treatment of this hydrolyzed extract, as described above, yields a highly purified benzene solution of the yellow pigments, while the green pigments remain in the water-soluble fraction.

The data so far obtained indicate that chlorophyll a becomes radioactive much faster than chlorophyll b. This suggests that chlorophyll a is a biosynthetic precursor of chlorophyll b. This result confirms earlier observations.[3] A similar observation has been made in higher plants. (Chlorophyll a was more radioactive than chlorophyll b even after 24-hr exposure to $C^{14}O_2$).[4]

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[3] Ulrich Blass, in Chemistry Division Quarterly Report, UCRL-3629, Jan. 1957, p 34.

[4] Shlyk, Godnev, Rotfarb, and Liakhnocich, Doklady Akad. Nauk SSSR 113, 1324 (1957).

In the carotenoid series, three groups with high, medium, and low radioactivity can be distinguished. The first group consists of the two carotenes; the second group contains xanthophyll and violaxanthin;[5] and the third group contains xanthophyll epoxide and the unidentified monepoxy carotenoid C4 (possibly antheraxanthin). Each group of higher molar radioactivity represents a biosynthetic precursor for the group with the next lower radioactivity, while members of a given group are in equilibrium with one another. From these preliminary experiments the following tentative scheme for the path of carbon through the pigments of Scenedesmus may be suggested:

$$\xrightarrow{C^{14}} \left\{ \begin{array}{c} \beta\text{-carotene} \\ \uparrow\downarrow \\ \alpha\text{-carotene} \end{array} \right\} \xrightarrow{C^{14}} \left\{ \begin{array}{c} \text{Xanthophyll} \\ \\ \text{Violaxanthin} \end{array} \right\} \xrightarrow{C^{14}} \left\{ \begin{array}{c} \text{Xanthophyll epoxide} \\ \uparrow\downarrow \\ \text{C4 (antheraxanthin ?)} \end{array} \right.$$

$$\xrightarrow{C^{14}} \text{chlorophyll } \underline{a} \qquad \xrightarrow{C^{14}} \text{chlorophyll } \underline{b}$$

[5] Sapozhnikov, Krasovskaya and Mayevskaya, Doklady Akad. Nauk SSSR 113, 465 (1957), find a conversion of xanthophyll into violaxanthin in the dark, and the reverse in the light, in higher plants.

INCORPORATION OF IRON-59 INTO ALGAE LIPIDS

Ulrich Blass

As a continuation of earlier work by Jean Bourdon,[1] the incorporation of iron into the lipids of algae has been investigated further.

Chlorella algae were inoculated in 103 ml iron-deficient Myer's medium and grown with 110 μC iron Fe^{59} citrate for three days. The algae were centrifuged and yielded 1.4 ml wet-packed cells. These were extracted twice with 20 ml boiling methanol. The extract was diluted with 10 ml benzene and 100 ml 5% aqueous sodium chloride solution and was separated by centrifugation. Planchets were made and counted (aqueous aliquots were diluted 10 times with alcohol).

Portion	Total Fe^{59} Activity (cpm)
Supernatant of the algae	77.0×10^6
Extract, water layer	2.94×10^6
Extract, benzene layer	0.42×10^6

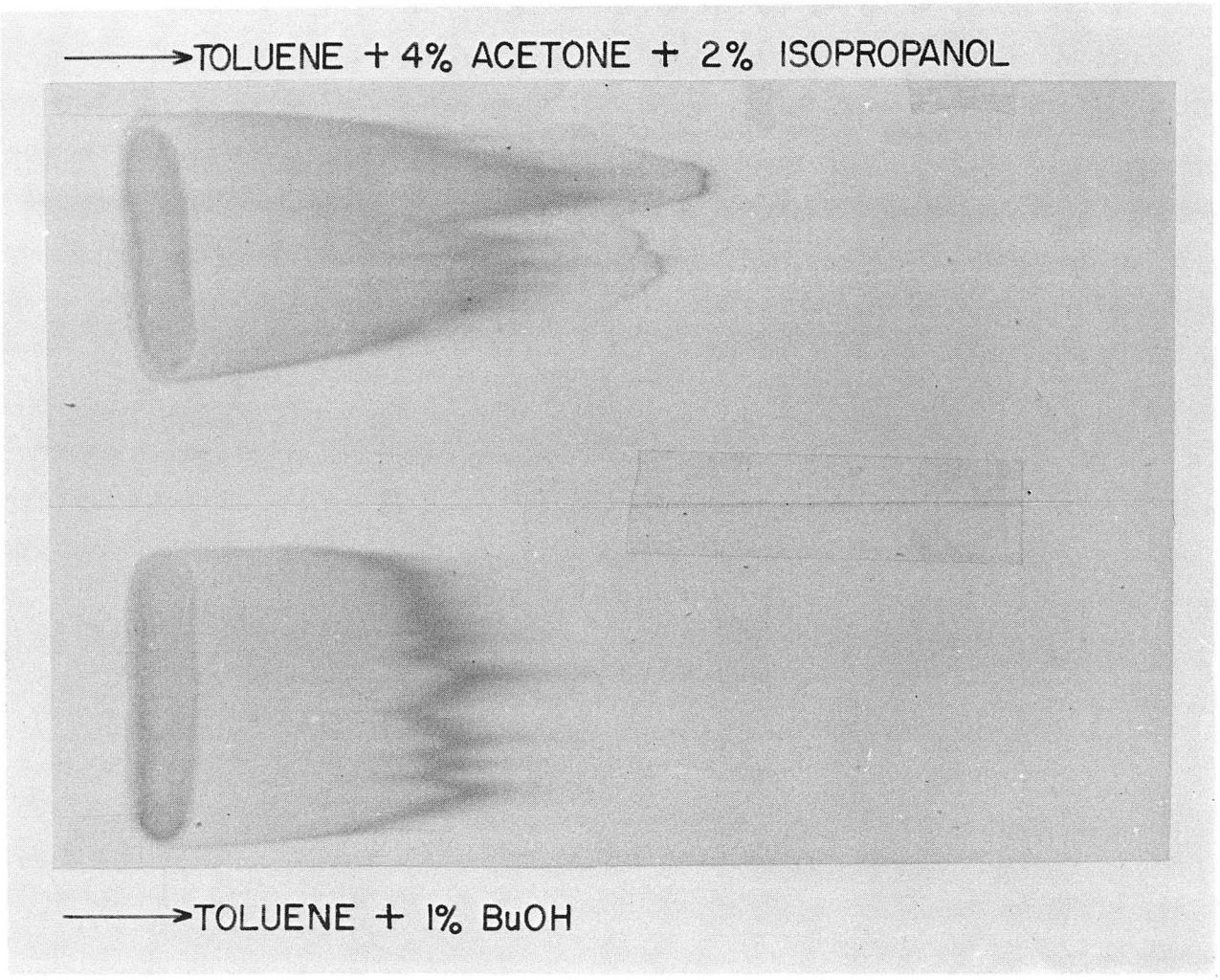
The counting efficiency for Fe^{59} was found to be 1.0×10^6 cpm/μC. From this figure the Fe^{59} content of the centrifuged cells can be calculated as $(110 - 77) = 33 \times 10^6$ cpm, which indicates that one-third of the Fe^{59} has not been extracted from the cells by methanol.

Ascending one-dimensional paper chromatograms were made by use of 100 λ of the benzene extract each with these solvent mixtures: toluene + 4% acetone + 2% isopropanol, and toluene + 1% n-butanol. The chromatograms were exposed to x-ray films for three weeks. Their autoradiographs (Fig. 12) show some radioactivity on the origin of the chromatogram, whereas most of the Fe^{59} can be found in a zone between the phospholipids and the mono-acylglycerides. This zone has been isolated in an ascending preparative paper chromatogram on Whatman No. 3 MM paper on which 2 ml of the benzene extract with about 55,000 cpm was applied as a streak to the base line of the whole sheet. The iron zone was cut out and extracted four times with 5 ml boiling methanol–benzene, 4:1. The extracts were diluted with 5% aqueous sodium chloride solution and separated by centrifugation. The benzene solution contained no more than 2300 cpm and showed the following spectral data:

Volume	$\log I_o/I$	λ_{max}	Calculated amount of porphyrin
4.0 ml	0.04	662 mμ	1.9×10^{-9} mole
	0.15	437 mμ	

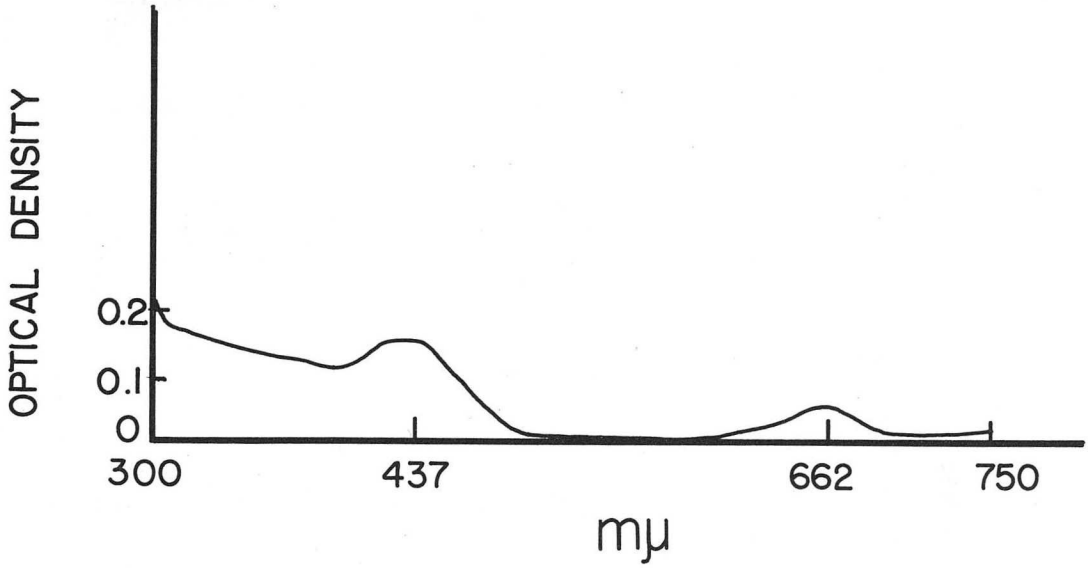
Figure 13 shows the absorption spectrum of the eluted iron zone, which is typical for a green porphyrin pigment. This pigment deserves further investigation, although it was easily extracted off the paper while only a minor part of the Fe^{59} was extracted at the same time.

[1] Jean Bourdon, in Chemistry Division Quarterly Report, UCRL-3351, April 1956, pp 52-53.
Calvin



ZN-1809

Fig. 12. Autoradiographs of paper chromatograms from the lip fraction containing Fe^{59}. Chlorophyll a and b were in th nonradioactive area ahead of the Fe^{59}.



MU-14048

Fig. 13. Absorption spectrum of the eluted chromatogram zone carrying Fe^{59}.

POSSIBLE EFFECT OF THE SPECIFIC ACTIVITY OF $C^{14}O_2$ UPON PHOTOSYNTHESIS. I

Christian van Sumere

In previous Quarterly Reports we have discussed the effect of gamma radiation from a Co^{60} source on the growth and metabolism of Chlorella pyrenoidosa.[1,2] The total $C^{14}O_2$ fixation over a 3-minute period was not significantly altered one hour after gamma radiation, even at doses up to 10^6 rep. On the other hand, the work of Boichenko and Zakharova suggests a possible unique effect upon photosynthesis due to the presence of $C^{14}O_2$.[3,4] They report that normal photosynthesis can occur only at very low levels of $C^{14}O_2$ (0.004% C^{14}) and that in the presence of 0.72% $C^{14}O_2$, photosynthesis is reduced to approximately 1/40 of the normal value over a 10-minute period. Unless the result they observed is a unique one due to C^{14} (either an isotope effect or an effect due to the type and location of the beta radiation) it is difficult to reconcile the effects reported from $C^{14}O_2$ with those obtained from gamma radiation.

The experiments described below were done to test the possible consequence of varying the specific activity of $C^{14}O_2$ upon the fixation of $C^{14}O_2$.

Experimental Procedure

An algae suspension (Chlorella pyrenoidosa) from the center-tube culture (24 hours' light) was centrifuged and the packed cells were diluted to a 1% suspension in distilled water. Two ml of this 1% suspension were then pipetted into flattened test tubes (one set of three long lollipops). The algae were illuminated by photoflood lamps in all experiments.

One percent CO_2 in air was bubbled through the tubes for 10 minutes. Then the aerating tube was quickly removed from the first lollipop and immediately thereafter 100 λ of $NaHC^{14}O_3$ (0.026 M, 400 μC/ml, 15.4 mC/mmole, 38% C^{14}) was injected into it. The lollipop was shaken between the lights for 5 seconds to 3 minutes, and subsequently 8 ml of hot absolute alcohol was injected into the suspension to kill and to extract the

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[1] C. van Sumere and O. Holm-Hansen, in Chemistry Division Quarterly Report, UCRL-3710, March 1957, p 32.

[2] C. van Sumere and O. Holm-Hansen, in Chemistry Division Quarterly Report, UCRL-3836, June 1957, p 42.

[3] E. A. Boichenko and N. I. Zakharova, Biokhimiya (English translation) 21, 377 (1956).

[4] E. A. Boichenko and N. I. Zakharova, Conference of the Academy of Sciences of the USSR on The Peaceful Uses of Atomic Energy, June 1955 (English Translation) 1956, p 121.

algae. Parallel experiments were carried out using $NaHC^{14}O_3$ diluted ten- and one hundred-fold.* Total fixation was measured by plating aliquot portions of the alcohol extract on planchets and counting on a Nuclear-Chicago Model D-47 counter with a Model C-110A automatic sample changer.

Tables XVI and XVII present the results of short-term photosynthesis experiments in which the $C^{14}O_2$ specific activity was varied 100-fold. For very short photosynthesis (40 sec) the ratios of C^{14} incorporated at high specific activity to that incorporated at low specific activity were less than theory in many experiments. For photosynthesis experiments of 100 to 180 seconds' duration this ratio was larger than expected. However, the ratios obtained were not very reproducible, and further experiments must be done with refinements of experimental technique before a definitive answer can be given as to the possible effects of C^{14} upon photosynthesis. It seems certain, however, that no inhibition of photosynthesis such as that reported by Boichenko[3, 4] has been found in our experiments.

*Two 100-λ portions of $NaHC^{14}O_3$, 0.026 M, 400 μC/ml were diluted with 0.900 ml and with 9.90 ml of 0.026 M $NaHC^{12}O_3$ prepared in the same manner as the radioactive solution. Aliquot portions (100 λ) of these diluted solutions, containing 40 μC/ml and 4 μC/ml, were used for the 10-fold and 100-fold dilution experiment.

Table XVI

Effect of specific activity of the added $HC^{14}O_3^-$ upon the total fixation of Chlorella

	Time of exposure to $HC^{14}O_3^-$ (sec photosynthesis)	Specific activity of $NaHC^{14}O_3$ (mC/mmole)	Total cpm (corr. for background)	Experimental Ratio A:C (Theory=100)	A:B (Theory=10)	B:C (Theory=10)
A	20[a]	15.4	584,000	37	6.7	
B		1.54	87,000			5.5
C		0.154	15,850			
A	30[b]	15.4	3,240,000	72	8.6	
B		1.54	380,000			8.5
C		0.154	45,000			
A	30[c]	15.4	1,560,000	41	6.9	
B		1.54	227,000			6.0
C		0.154	38,000			
A	180[c]	15.4	8,800,000	135	14.9	
B		1.54	588,000			9.0
C		0.154	65,300			
A	30[c]	15.4	1,410,000	41	6.5	
B		1.54	218,000			6.4
C		0.154	34,200			

[a]Experiment 1: Flattened test tubes containing 2 ml of a 1% Chlorella suspension were illuminated with photoflood lamps. 1% CO_2 in air was bubbled through the suspension for 10 minutes. Aliquot portions (100 λ) of $NaHC^{14}O_3$ (0.026 M) containing 40, 4, and 0.4 μC of the indicated specific activity were added and photosynthesis was continued for the indicated time. The algae were killed by hot alcohol and 250-λ aliquot portions (total volume 12 ml) were plated. The times to count 20 x 256 counts on the D-47 flow counter were determined, and the total cpm present calculated after correction for the background (24 cpm). This experiment was done with H. Simon.

[b]Experiment 2: Same as Experiment 1, except round lollipops containing 8 ml of a 2% Chlorella suspension were used. Total final volume was 50 ml; 50-λ aliquot portions were plated. This experiment was done with H. Simon.

[c]Experiment 3: Same experimental conditions as in Experiment 1. Time to count 10 x 256 counts was determined.

Table XVII

Possible effect of the specific activity of $NaHC^{14}O_3$ upon photosynthesis for several time intervals

	Time of exposure to $HC^{14}O_3^-$ (sec photosynthesis)	Specific activity of $NaHC^{14}O_3$ (mC/mmole)	Total cpm (corr. for background)	Experimental Ratio A:C (Theory=100)	Experimental Ratio A:B (Theory=10)	Experimental Ratio B:C (Theory=10)
A	5	15.4	341,000	46	12.9	
B		1.54	26,400			3.4
C		0.154	7,480			
A	10	15.4	519,000	50	12.0	
B		1.54	43,000			4.2
C		0.154	10,300			
A	20	15.4	705,000	29	4.0	
B		1.54	175,000			7.2
C		0.154	24,200			
A	40	15.4	1,760,000	66	8.2	
B		1.54	214,000			8.1
C		0.154	26,600			
A	80	15.4	3,660,000	92	9.1	
B		1.54	405,000			10.1
C		0.154	40,000			
A	100	15.4	6,870,000	163	16.9	
B		1.54	406,000			9.7
C		0.154	42,200			

Experiment 4: Experiment carried out similarly to Experiment 1. Total volume of killed *Chlorella* suspension was 11.0 ml. The aliquot portions counted were 100 λ for 5-second photosynthesis; 40 λ for 10-, 20-, and 80-second photosynthesis; and 20 λ for 40- and 100-second photosynthesis. The times to count 2560 counts on the D-47 counter were determined for the planchets prepared from 5-, 10-, 20- and 40-second photosynthesis experiments, and the times to count 10,240 counts were determined for the planchets from the 80- and 100- second photosynthesis experiments. Calculated after correction for the background of 24 cpm.

POSSIBLE EFFECT OF THE SPECIFIC ACTIVITY OF $C^{14}O_2$ UPON PHOTOSYNTHESIS. II

Osmund Holm-Hansen

In consideration of the importance of the findings described by C. van Sumere in the preceding paper in this Quarterly Report, it was deemed necessary for another person to try to reproduce these effects. In this way it was hoped to eliminate any systematic error that might be characteristic of one person's methods, and thus to shed light on whether or not these effects represent an actual physiological response by the algae.

For all the experiments except the first, Chlorella pyrenoidosa from the center-tube culture were used; this is the culture that was growing under continuous light, and with the usual CO_2 and nutrient-solution supply. The elongated, flattened test tubes were used in sets of three throughout, and contained either 2.0 or 3.0 ml of a 1% algae suspension in distilled water. These tubes were held in a water-cooled bath both during the adaptation period and after the addition of the $NaHC^{14}O_3$. During the adaptation period, two photoflood lamps were used to illuminate the algae and 1% CO_2 in air was rapidly bubbled through the algal suspensions. At the end of the adaptation period, the aeration tube was removed and the tube manually shaken by one person while another person injected the $NaHC^{14}O_3$, started the stopwatch, and got the alcohol ready for killing the algae. Care was taken to insure that the aeration in the tubes did not stop before the aeration tube was removed. The same volume (100 λ) of $NaHC^{14}O_3$ (0.0266 M) was injected into each of the tubes, but its specific activity varied; it was 0.15 mC/mmole, 1.5 mC/mmole, and 15 mC/mmole (in the order in which the photosynthesis tests were made).*

As the adaptation period started at the same time for all three tubes, it was longer for the third tube than for the first tube by the length of time it took to get the first two tubes to the point of addition of the hot alcohol. It is not known whether this time difference is significant or not. In the first three experiments described, the 30-second photosynthesis tests were run first, followed by the 3-minute tests (only three tubes can be adapted at one time). During this time delay, the 1% algal suspension was kept in an Erlenmeyer flask. In the last experiment described, the algal suspension was continuously aerated vigorously with 4% CO_2 in air. Immediately before the start of the adaptation period for the different time tests (from 6 seconds to 3 minutes) the algal suspension was pipetted directly into the test tubes. After the alloted time of exposure to $HC^{14}O_3^-$ the algae were killed by rapid injection of hot absolute ethanol. Measurement of the total fixation was done by making planchets in the usual fashion and counting with either the Scott tube or a Nuclear-Chicago Model D-47 counter with a Model C-110A automatic sample changer.

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*The stock $NaHC^{14}O_3$ solution was 0.0266 M and had activity of 400 μC/ml; 15.0 mC/mmole. An aliquot of 100 λ of this was used directly for the high-specific-activity samples. A 0.0266 M solution of $NaHC^{12}O_3$ was prepared (from freshly boiled and cooled double-distilled water) and suitable portions of these two solutions were mixed so that the mixed solutions contained 40 μC/ml and 4.0 μC/ml. The specific activity of C^{14} in the algal suspensions was, therefore, in the ratio 1:10:100.

The data and experimental conditions of Experiments I, II, and III are summarized in Tables XVIII, XIX, and XX. It is seen that the experimental ratios fluctuate above and below the theoretical ratios, but in general tend to be higher, which is in contrast to the results presented by C. van Sumere. The data from Experiment IV, summarized in Table XXI and plotted in Fig. 14, show that the magnitude of variation in total C^{14} fixation by algae exposed to the three different specific activities of $HC^{14}O_3$ is not very great, and most likely is within the experimental error. It is worth noting in this experiment that the total fixation of C^{14} in the samples with the highest specific activity was very high when calculated on the basis cpm/min photosynthesis/ml packed cells. The findings on this basis, and the order in which the photosynthetic tests were made, may be summarized as follows:

Exposure (sec)	6	10	15	40	60	80	120	180
Order in which tested	2	8	3	4	7	1	5	6
cpm x 10^6	86	92	106	102	93	85	96	88

It is seen that the time of the test (whether done first or last) probably had no significant effect. The discrepancy between these results and those of C. van Sumere is pronounced, but at this time no satisfactory answer can be given to explain the difference.

Further experiments on this problem are now under way and will be reported in the next quarterly report.

Table XVIII

Experiment I: Effect of specific activity of the added $HC^{14}O_3^-$ upon the total fixation by Chlorella[a]

	Time of exposure to $HC^{14}O_3^-$	Specific activity of $NaHC^{14}O_3$ added (mC/mmole)	Aliquot on planchet (total vol, 10.0 ml)(λ)	Total cpm minus background[b]	Total cpm in sample, average	Experimental ratio A:C (theory=100)	Experimental ratio B:C (theory=10
A	25 sec	15.0	250 250	8578 8541	343,000	90.5	
B	25 sec	1.5	250 250	937 924	37,250		9.85
C	25 sec	0.15	250 500 500	99 178 193	3,793		
A	3 min	15.0	100 100	19,400 18,550	1,897,000	98	
B	3 min	1.5	100 100	1,618 1,627	162,250		8.35
C	3 min	0.15	100 200 300	192 372 611	19,400		

[a]Experimental conditions: Chlorella from the third tube were used. (This culture is on a 12-hour-light–12-hour-dark cycle.) The cells were adapted with 1% CO_2 in air for 40 min. From the time the aeration tube was lifted out of the algal suspension, there was a total time delay of 15 sec until the $HC^{14}O_3^-$ was added. Each lollipop contained 2.0 ml of suspension.

[b]Each planchet was counted four times with the Scott tube; the background was measured between every planchet.

Table XIX

Experiment II: Effect of specific activity of the added $HC^{14}O_3^-$ upon the total fixation by Chlorella[a]

	Time of exposure to $HC^{14}O_3^-$	Specific activity of $NaHC^{14}O_3$ added (mC/mmole)	Aliquot on planchet (total vol, 15.0 ml)(λ)	Total cpm minus background[b]	Total cpm in sample, average	Experimental ratio A:C (theory=100)	Experimental ratio B:C (theory=10)
A	30 sec	15	100 100	1434 1405	213,000	130	
B	30 sec	1.5	200 200	277 300	21,650		13.2
C	30 sec	0.15	400 400	46 42	1,640		
A	3.5 min	15	50 50	6136 5506	1,745,000	174	
B	3.5 min	1.5	100 100	918 1023	145,000		14.5
C	3.5 min	0.15	100 200	60 147	10,000		

[a]Experimental conditions: Chlorella from the center tube were used. Each test tube contained 2.0 ml of algal suspension. The $HC^{14}O_3^-$ was added immediately after removal of the aerating tube. Adaptation time was 10 min.

[b]Each planchet was counted four times with the Scott tube; the background was measured between every planchet.

Table XX

Experiment III: Effect of specific activity of the added $HC^{14}O_3^-$ upon the total fixation by Chlorella[a]

	Time of exposure to $HC^{14}O_3^-$	Specific activity of $NaHC^{14}O_3$ added (mC/mmole)	Aliquot on planchet (total vol, 11.0 ml)(λ)	Total cpm minus background	Total cpm in sample, average	Experimental ratio A:C (theory=100)	Experimental ratio B:C (theory=10)
A	30 sec	15	250	10,860	471,000	155	
			250	10,550			
B	30 sec	1.5	250	880	38,900		12.8
			250	887			
C	30 sec		500	137	3.030		
			500	138			
A	3 min	15	40	8,195	2,315,000	137	
			40	8,650			
B	3 min	1.5	75	1,455	211,000		12.5
			75	1,416			
C	3 min	0.15	150	216	16,850		
			150	241			

[a]Experimental conditions were the same as for Experiment II.

Table XXI

Experiment IV: Effect of specific activity of added $HC^{14}O_3^-$ upon the total fixation by Chlorella[a]

	Time of exposure to $HC^{14}O_3^-$ (sec)	Specific activity of $NaHC^{14}O_3$ added (mC/mmole)	Aliquot portion on planchet (total vol, 25 ml) (λ)	Net cpm/ml average[b]	Experimental Ratio A:C (theory=100)	Experimental Ratio B:C (theory=10)	Experimental Ratio A:B (theory=10)
A	6	15	100	11,000	77		12.6
B		1.5	500	870		6.1	
C		0.15	1000	143			
A	10	15	100	20,100	95		12.1
B		1.5	100	1,661		7.8	
C		0.15	500	211			
A	15	15	100	35,140	117		10.3
B		1.5	250	3,420		11.4	
C		0.15	500	300			
A	40	15	100	92,000	101		9.6
B		1.5	250	9,538		10.5	
C		0.15	500	907			
A	60	15	50	132,160	91		8.6
B		1.5	50	15,300		10.7	
C		0.15	250	1,436			
A	80	15	50	146,370	91		7.9
B		1.5	250	18,496		11.2	
C		0.15	500	1,654			
A	120	15	50	256,190	94		7.3
B		1.5	100	34,895		12.8	
C		0.15	250	2,736			
A	180	15	40	396,360	103		8.6
B		1.5	50	46,290		12.0	
C		0.15	250	3,848			

[a]The same experimental conditions as in Experiments II and II were used, except that 3.0 ml of algal suspension was taken.

[b]Duplicate planchets were prepared. The time to count 2560 counts was determined for one (background 20.8 cpm) and the time to count 10,240 counts (background 18.5 cpm) was determined for the other on the Nuclear-Chicago Model D-47 counter with a Model C-110A automatic sample changer.

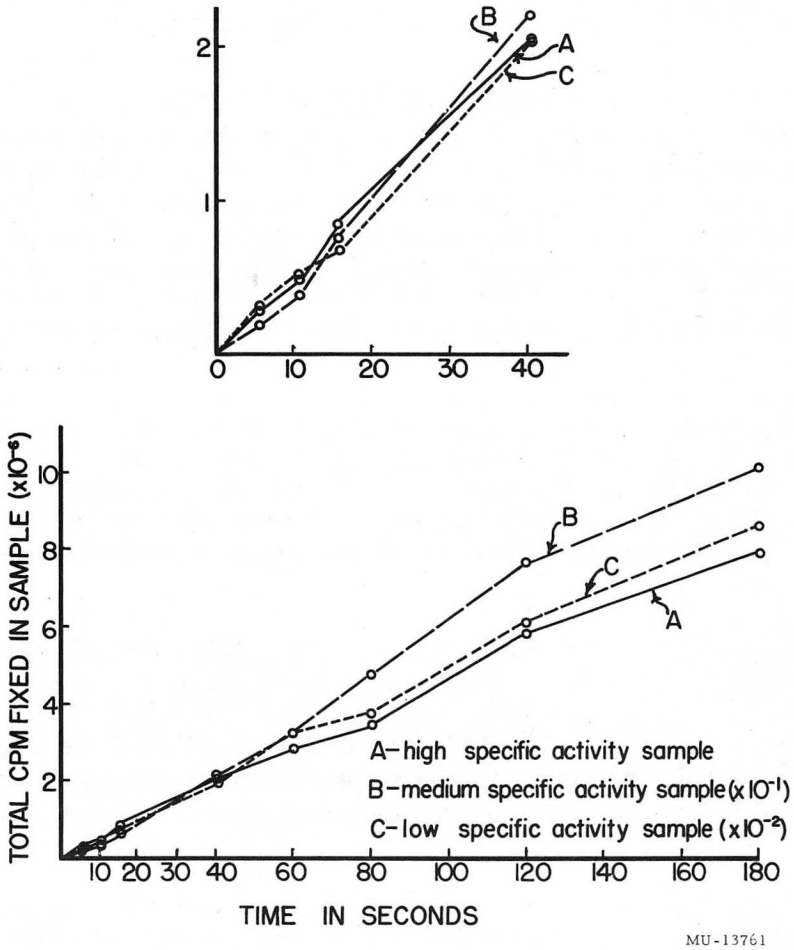

Fig. 14. Total fixation of C^{14}-bicarbonate by Chlorella, when exposed to three different specific activities of $HC^{14}O_3^-$. Data from Experiment IV. The specific activity of $NaHC^{14}O_3$ of A:B:C was in the ratio 100:10:1.

CYANIDE EFFECTS ON CARBON DIOXIDE FIXATION IN CHLORELLA

Joan M. Anderson and Duncan F. Shaw

The effect of cyanide on the $C^{14}O_2$ fixation in the alcohol-water-soluble fractions of Chlorella has been studied by Otto Kandler.[1] The essential features of his findings were the large increase in C^{14} activity in the diphosphates and the concurrent decrease of C^{14} activity in phosphoglyceric acid. Dephosphorylation of the diphosphate area gave two new spots exhibiting acid lactone behavior, in addition to other known sugars. Further work has been undertaken as described below.

Experimental Procedure

Chlorella pyrenoidosa that had been cultivated in the continuous-culture apparatus was centrifuged, washed, and resuspended to give a 1% suspension in distilled H_2O. Aliquots of 10 ml were used in the photosynthetic experiments, which were performed in a modified "lollipop," consisting of an open bulb (50 ml) connected to a long flat tube (10 ml) surrounded by a water jacket. The bicarbonate, cyanide, and alcohol were injected into the suspension through the upper opening, thus allowing the cells to be killed in the light.

The algae were pre-illuminated for 20 minutes; a continuous stream of 1% CO_2 in air was bubbled through during this period. At the commencement of the experiment the air stream was turned off, and the sodium bicarbonate was added. After two minutes' photosynthesis with stirring, the KCN was added, and after a further 15 seconds the algae were killed by the addition of boiling ethanol.

The experiments were of three types: (a) control photosynthesis experiment with $NaHC^{14}O_3$, designated as $C^{14}O_2$; (b) photosynthesis experiment with $NaHC^{14}O_3$ plus KCN, designated as $C^{14}O_2$, CN^-; (c) photosynthesis experiment with $NaCHO_3$ plus $KC^{14}N$, designated as CO_2, $C^{14}N^-$. The concentration of the radioactive material, when used, was the same as that of the nonradioactive material; the $NaHCO_3$ was 0.0013 to 0.00013 M and the KCN was 0.15 to 0.24 M.

Extracts were prepared for chromatography by centrifuging the suspension after removing an aliquot portion to estimate the total fixation. The extract was retained and the residue successively extracted with 20% ethanol (10 ml) and boiling water (10 ml). The extracts were combined, concentrated to approximately 4 ml in a rotating evaporator, and chromatographed on paper in the usual solvents. The diphosphate areas were cut out and eluted with water. The eluate was chromatographed in the Trudinger solvent system.[2] Further, the diphosphates were dephosphorylated by one of the following methods: (a) acid hydrolysis using HCl (1 N) in a sealed tube at 120°C for 16 hours; (b) enzymic hydrolysis at pH 5 (0.2 M acetate buffer) using Polidase-3 or acid prostatic phosphatase.

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[1] O. Kandler, in Chemistry Division Quarterly Report, UCRL-3710, March 1957, p. 8.

[2] P. A. Trudinger, Biochem. J. 64, 274 (1956).

Results

The general pattern of a radioautograph of a typical $C^{14}O_2$, CN^- experiment is rather similar to that obtained in the absence of cyanide. From a cursory examination it might be concluded that cyanide is acting as an enzyme poison, since cyanide has been widely used in biochemical systems as an inhibitor of such enzymes as tyrosinase and catalase. However, the radioautographs of typical CO_2, $C^{14}N^-$ experiments show a large number of new spots. The sole source of radioactivity here is cyanide, therefore all the spots are the products of the reaction of cyanide with material present in the algae.

Radioautographs of the dphosphorylated areas obtained from the above experiments, either by enzymic hydrolysis or acid hydrolysis, show large amounts of a material not present in experiments without cyanide addition. Since it has been demonstrated that cyanide participates in the formation of this material, the possibility was investigated that it might be produced by a cyanohydrin reaction on ribulose diphosphate (RuDP).[3] $KC^{14}N$ and RuDP yielded hamamelonic acid diphosphate, which was hydrolyzed to hamamenlonic acid.

A comparison of the electrophoretic behavior of the diphosphate area of the experiment $C^{14}O_2$, CN^- with synthetic hamamelonic acid diphosphate showed the two materials to be electrophoretically indistinguishable. Radioautographs of the diphosphates of both the $C^{14}O_2$, CN^- and CO_2, $C^{14}N^-$ experiments in the Trudinger solvent system[2] showed exact coincidence of the radioactivity with the reaction product of KCN plus ribulose diphosphate. Cochromatography in one dimension (butanol-propionic acid-water) showed the material from experiments $C^{14}O_2$, CN^- and CO_2, $C^{14}N^-$ to be identical with synthetic hamamelonic acid.

It has therefore been shown that the material appearing in photosynthesis experiments by the use of high concentrations of cyanide is hamamelonic acid diphosphate, the source of which is the cyanohydrin reaction on ribulose diphosphate.

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[3] P. R. Rabin, D. F. Shaw, N. G. Pon and J.M. Anderson, this report, p. 23.

DETERMINATION OF THE CONCENTRATION AND THE SPECIFIC ACTIVITY OF $KC^{14}N$

Joan M. Anderson

In order to determine the specific activity and concentration of $KC^{14}N$ prepared by R. M. Lemmon et al.[1] for use in in vitro and in vivo experiments,[2,3] it was necessary to develop satisfactory microanalytical methods.

The concentration of the cyanide was determined by a modification of the cyanogen bromide technique.[4] KCN (1 to 10 μg) was diluted in water to 1 ml, and saturated bromine water (0.5 ml) was added. The excess bromine was destroyed by the addition of $NaAsO_2$ (0.5 ml of 0.1 M). Pyridine (5 ml of a 25% solution in water containing 2 ml of concentrated HCl) was then added, followed by benzidine hydrochloride (0.2 ml of 2% solution in water). A red coloration was produced which intensified on standing. Samples of varying known concentrations of KCN were prepared, and the optical density at the λ_{max} (413 mμ) was taken at the same time interval after each sample had been prepared. Thus a standard curve was obtained from which the unknown $KC^{14}N$ concentration could be ascertained. It was necessary to redetermine the standard curve for every run, because it appeared to change slightly from time to time; a variety of factors could be causing this, such as temperature, pH, the amount of light the samples received, the time interval elapsed, and so on. Consistent results are obtainable, however, if the linear curve is determined each time.

For the specific activity determination it was necessary to prepare a nonvolatile solid. Since AgCN appeared unsatisfactory, Prussian blue, $Fe_4(Fe(CN)_6)_3$, was prepared. $KC^{14}N$(5λ) was diluted to 1 ml in water, and 250 λ of this solution added to KCN (250 λ of 1.0 M); the nonradioactive cyanide is used to give a satisfactory amount of precipitate. $FeSO_4$(40 λ of 1.0 M) is added to the cyanide to form potassium ferrocyanide, followed by $FeCl_3$ (50 λ of 1.0 M) to give a deep blue precipitate of Prussian blue. The mixture was warmed and two drops of HCl (5 M) added. The precipitate was washed four times with water (2 ml portions) by centrifugation, finely ground up with a glass rod, and made up to 2 ml in EtOH. Aliquot portions (25 λ containing approximately 0.1 mg) were plated out onto weighed planchets and counted. The cpm/mg Prussian blue were converted to cpm/mg KCN plus $KC^{14}N$. The specific activity of $KC^{14}N$ is obtainable from the ratio of KCN added to the original $KC^{14}N$.

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[1] R. M. Lemmon, B. Fingerman and H. Simon, in Chemistry Division Quarterly Report, UCRL-3836, June 1957, p. 18.

[2] B. R. Rabin, D. F. Shaw, N. G. Pon, and J. M. Anderson, in this report, p. 23.

[3] J. M. Anderson and D. F. Shaw, in this Chemistry Division Quarterly Report, p. 63.

[4] W. N. Aldridge, Analyst. 69, 262 (1944).

INTENSIFICATION OF X-RAY-FILM IMAGES BY BLUE TONING

Karl Lonberg-Holm and V. Moses

When x-ray film is used to obtain an autoradiogram of a chromatogram, the period of exposure necessary to obtain a satisfactory blackening of the film on subsequent development is proportional to the radioactivity of substances on the chromatogram. Exposure time may vary from a few minutes for some preparative samples to weeks or months for very weakly active samples. Any reasonably simple method of speeding this process is desirable, especially in cases in which a long period of film exposure is necessary.

Upon consultation, a representative of E. I. duPont de Nemours and Company suggested three possible methods of increasing the sensitivity of x-ray film. One method, which has not been investigated in this laboratory, consists of using film without the normal antiabrasion surface layer of gelatin with which x-ray film is normally covered. This would permit the chromatogram to be brought into closer contact with the sensitive emulsion of the film. Absorption of weak radiation in the gelatin layer would be obviated and a stronger film image would be produced in a given time.

Two methods, discussed in more detail below, were investigated. Intensification of the film image after development and fixation increased the sensitivity of the film 3 to 5 times over the usual method of development.

Intensification by Illumination

The response of photographic emulsion to incident radiation (light, radioactive particles, etc.) is not linear at very low levels, and there is a threshold value of incident radiation below which no usable image is produced. It should therefore be possible to intensify the image produced on an x-ray film by a chromatogram of radioactive substances by exposure of the film before the development to a light source of such color and intensity that it would be just insufficient to fog the background. Under these conditions areas already containing a latent image from the chromatogram would be brought above the threshold value and would be correspondingly "fogged" by the light exposure, while the background would be unaffected by the particular light source used. Attempts to achieve this type of intensification, using a blue-green light source, were ineffective and the method was not pursued further.

Intensification by Toning

This method consists of immersing the developed, fixed, and washed film in a toning solution for the desired period, followed by washing to remove the excess toning solution. During toning, iron salts in the solution convert the weak silver image of the radiograph into an intense blue image consisting of ferric ferrocyanide and a mixture of silver ferrocyanides and ferricyanides. The blackened areas on the film are turned deep blue, and some blue color is also produced in the background. The most pronounced effect is seen if the intensified film is viewed through a red filter. A chromatogram of radioactive glucose (sufficiently active to expose a film in

a few minutes) was exposed to Dupont 507E blue-sensitive x-ray film, for 3 min and 10 min. Two films were exposed for 3 min, one of which was intensified with the blue-toning solution. Subsequent examination of the film showed that exposure of the film for 3 min followed by blue toning and viewing through a red glass produced a stronger image with a greater wealth of detail than the film exposed for 10 min and not subsequently intensified. The sensitivity ("speed") of the film was thus increased over 3.3 times, probably to a value of about 5 times, when compared with the usual developing procedure. Blake, Roderick, and Umberger[1] found a relative film speed 6 times as great under these conditions.

Technique of Blue Toning

Formula:	Solution A	
	Water (about 20°C)	750 ml
	Diglycolic acid(α, α'-oxy-diacetic acid)	60 g
	Sodium hydroxide	36 g
	Solution B	
	Water (about 20°C)	100 ml
	Ferric nitrate · 9 H_2O	14 g
	Potassium fluoride · 2 H_2O	1 g
	Solution C	
	Water (about 20°C)	100 ml
	Potassium ferricyanide	5 g
	Sodium nitrite	1 g
	Total Volume	1000 ml

Solutions A, B, and C are prepared by adding the constituents in the order given above. Solution B is added to Solution A in 2 min with stirring. Solution C is added to Solution A plus B in 2 min with stirring. Stir 1 minute.

The sodium hydroxide and diglycolic acid act as a buffer and the final pH should be about 5. Potassium fluoride acts as an antifogging agent, and sodium nitrite as an anticorrosion agent. The solution is reported to be stable in stoppered amber-colored bottles for at least six months.

After washing, the film is totally immersed in the intensifying solution at 20°C for 2 to 8 min, depending on the state of the solution and the degree of intensification required. It may be removed during this period for inspection; the process is carried out in normal room lighting. The films are washed in running water for not more than 5 min and dried in the usual way. Dry films may be intensified after a preliminary soaking in water for 20 to 30 min to soften the gelatin covering.

[1] R. K. Blake, J. F. Roderick, and J. Q. Umberger, "Intensification of Radiographs by Toning," E. I. duPont de Nemours and Company, Inc., Photo Products Department, Wilmington, Delaware.

PHOTOCONDUCTIVITY AND SEMICONDUCTIVITY OF ORGANIC COMPOUNDS WITH CONJUGATED RING SYSTEMS

David Kearns

Photoconductivity and semiconductivity in numerous organic dyes possessing a conjugated ring system have been found by a number of investigators.[1-5] Eley and Parfitt have suggested there is a correlation between the number of π bonds, the presence of free radicals, and the conductivity of these conjugated ring compounds.[6] Among these compounds the phthalocyanins are of special interest because of their close similarity in structure to chlorophyll. The existence of free radicals[7] and polymorphism[8] in phthalocyanins increases the interest in these compounds.

Phthalocyanins

A detailed study of phthalocyanins is now being carried out to find what relationships exist between their photo-and semiconductivity and (a) free-radical content, (b) polymorphic structures as determined by x-ray diffraction, (c) solution and solid absorption spectra, and (d) impurity content and nature of the gases adsorbed on the surfaces of the compounds. These points are discussed in more detail below.

An apparatus has been constructed and is now being used to measure the dc semiconductivity of the polycrystalline phthalocyanin. During measurements samples are compressed between the two measuring electrodes to pressures up to 250 kg/cm^2 in a chamber that can be evacuated to a gas pressure of 10^{-4} mm Hg. Temperatures in the chamber may be varied from approximately -50°C to 500°C. A similar apparatus is to be constructed for photoconductivity measurements.

Ingram and co-workers have reported free radicals in phthalocyanin that appear to be due to a one-equivalent oxidation of the phthalocyanin.[7] We find what seems to be the same radical, and an additional one in much lower concentration. X-ray diffraction of various samples indicates, however, that these free radicals are related to the polymorphism of the phthalocyanins. Various extractions with basic dimethyl formamide and quinoline have been used to purify the crude phthalocyanin and remove the free radicals. Crude experiments have been made to find out if the electron spin resonance is photo-sensitive, but with negative results. The temperature dependence of the spin resonance will also be measured to further characterize the nature of the free radical.

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[1] H. Akamatu and H. Inokuchi, J. Chem. Phys. 20, 1481 (1950).

[2] D. C. Northrup and O. Simpson, Proc. Roy. Soc. (London) B67, 892 (1954).

[3] Bree, Carswell, and Lyons, J. Chem. Soc. 1728 (1955).

[4] D. E. Fielding and F. Gutman, J. Chem. Phys. 26, 411 (1957).

[5] Compton, Schneider, and Waddington, J. Chem. Phys. 27, 160 (1957).

[6] D. D. Eley and G. D. Parfitt, Trans. Faraday Soc 51, 1529 (1955).

[7] P. George and D. J. E. Ingram, J. Am. Chem. Soc. 79, 1870 (1957).

[8] F. W. Karasek and J. C. Decius, J. Am. Chem. Soc. 74, 4716 (1952).

Absorption spectra have been taken of H_2SO_4 solutions of metal-free phthalocyanin, and will be taken on Nujol suspensions of the phthalocyanin.

The effect of impurities such as metallic phthalocyanin on the conductivity as well as the electric spin resonance will be studied. Adsorbed gases on the surface of the phthalocyanin may have a pronounced effect on conductivity; for this reason measurements are being made in vacuo, where gas content may be controlled to some degree.

Other Organic Compounds

The electron spin resonance of thirty other compounds (in addition to phthalocyanin) with conjugated ring systems was measured, and free radicals were detected in fifteen. Free radicals were also formed by mixing together the compounds that would give one-equivalent oxidation and reduction of each other. A program similar to the one for phthalocyanin is planned for these compounds.

A possible outcome of this investigation of free radicals in photoconductivity of organic compounds might be the development of a polycrystalline organic solar battery. For this purpose a vacuum system has been constructed and used to sublime thin films of organic compounds. As yet attempts to produce a multilayered organic solar battery have been unsuccessful.

HUMAN METABOLISM OF MORPHINE-N-METHYL-C^{14}

Henry Rapoport, Mohindra Chadha,[1] and Melvin Look

The only published work on human metabolism of therapeutic doses of morphine is that by Elliot et al.[2] They used morphine-N-methyl-C^{14} and measured the radioactivity eliminated without regard to the nature of the compounds excreted. Other work on the human metabolism of morphine has been limited to massive dosage. Unspecific colorimetric and other identification techniques for morphine were used. This report describes work that is being undertaken in which normal humans were administered therapeutic doses of morphine-N-methyl-C^{14}; morphine and its metabolites were isolated from the urine.

Each of ten male volunteers was injected intramuscularly[3] with 1 ml of pyrogen-free aqueous solution containing 11.3 mg morphine-N-methyl-C^{14} hydrochloride with a total activity of 1.0 μcurie per milliliter.[4] Urine was collected for 24 hours from the ten volunteers and an additional sample was obtained after the 24-hour period. The urine was pooled, concentrated, and fractionated according to the scheme in Fig. 15.

Preliminary work has shown that the morphine will be quantitatively extracted and separated from the approximately 600 g of solid in the 1.5 liters of urine concentrate. Studies by Elliot et al.[2] showed that the majority of radioactivity of morphine-N-methyl-C^{14} was present in the urine. Fraction A-I, containing the water-soluble morphine conjugates, was hydrolyzed with hydrochloric acid and was subjected to the above scheme again; the resulting fractions are called H-II to H-IX. A general idea of the radioactivity of the various fractions was given by direct counting. More accurate wet-combustion–barium-carbonate counting techniques are being made. The total activities of the various fractions are given in Table XXII.

The appearance of activity in Fractions V and H-V is surprising, as these are the nonmorphine organic fractions. Morphine is being purified from Fractions VII and H-VII and its specific activity is being determined in order to indicate if any transmethylation occurred. Fractions VIII and H-VIII are being examined for normorphine in order to determine if any demethylation occurred. The purification and identification of materials are being continued on the other fractions, and results will be given in a later report.

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[1] Department of Chemistry, University of California; present address, New Delhi, India.

[2] Elliot, Tolbert, Adler, and Anderson, Proc. Soc. Exptl. Biol. Med. 85, 77 (1954).

[3] By Dr. H. W. Elliot, University of California Medical Center, San Francisco.

[4] M. Look and H. Rapoport, in Chemistry Division Quarterly Report, UCRL-3836, June 1957, p 19.

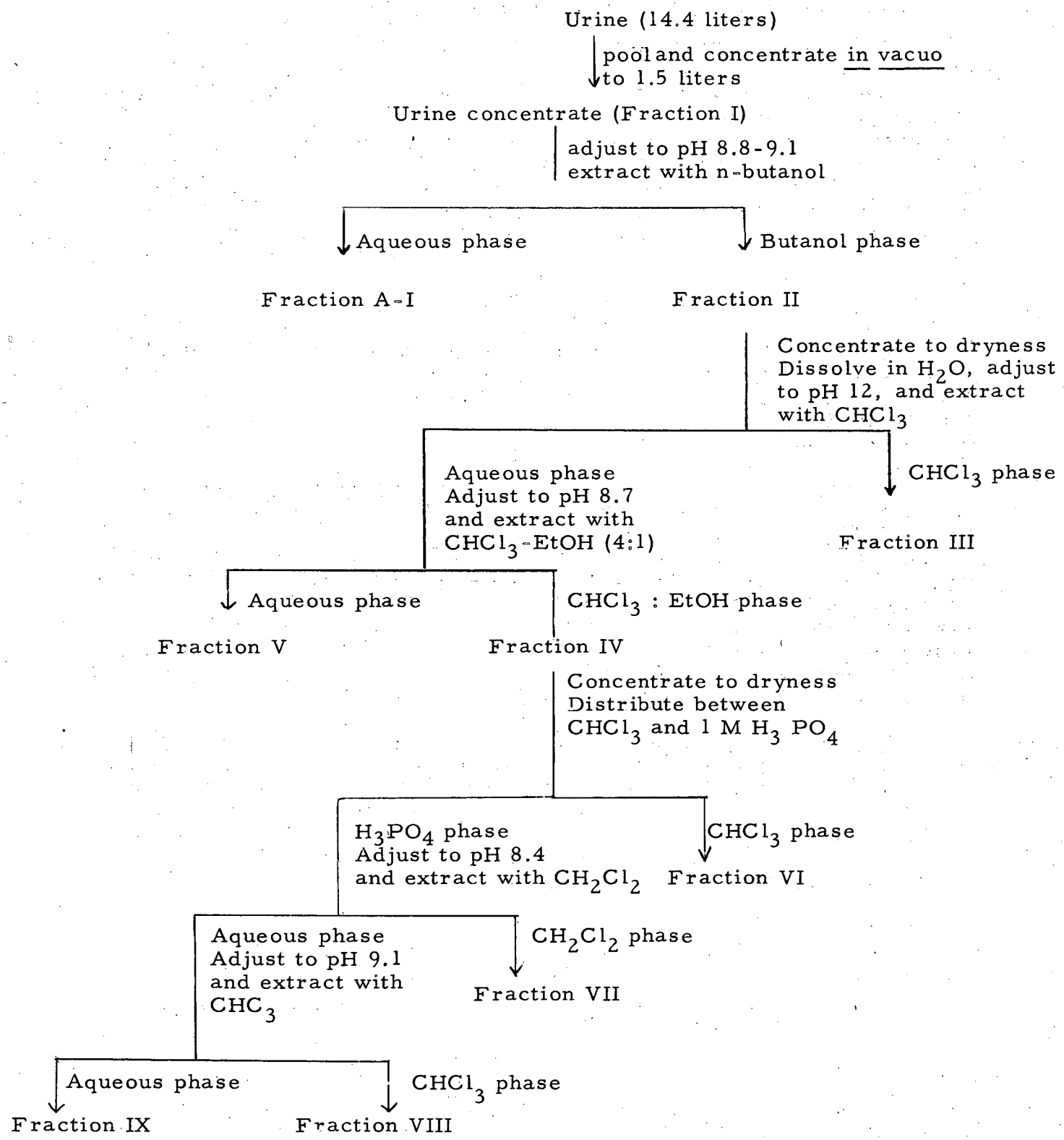


Fig. 15. Fractionation procedure for morphine derivatives in human urine.

Table XXII

Activity of Urine Fractions. Figures in parentheses were determined after combustion to CO_2 and plating as $BaCO_3$, and therefore are more reliable. Other activity determinations were by direct-plating techniques and are less reliable owing to interfering solids.

Fraction No.	Unhydrolyzed	Hydrolyzed (H) Fractions
I	7,400,000 dpm (13,700,000)	-
A-I	3,700,000 (10,200,000)	-
II	1,400,000 (2,190,000)	6,000,000 dpm (7,530,000)
III	No activity	No activity
IV	2,300,000	7,700,000
V	340,000	400,000
VI	No activity	No activity
VII	1,600,000	5,300,000
VIII	No activity	No activity
IX	No activity	No activity

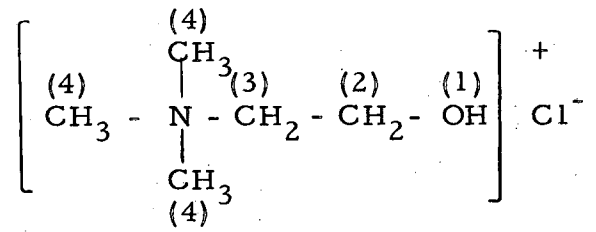
THE SELECTIVE DEUTERATION OF CHOLINE CHLORIDE

Robert Lindblom

An attempt to study the chain decomposition of crystalline choline chloride by means of electron spin resonance (ESR) is discussed in an earlier report.[1] The complexities of the spectra made interpretation very uncertain. Since most of the hyperfine structure in the ESR spectrum results from radical interaction with adjacent protons, replacement of the protons with deuterons generally eliminates this structure. This results from the fact that deuterons have only about 1/3 as much nuclear magnetic moment as protons and only about 1/7 as much signal response. For this reason individual deuteration of the distinguishable proton sites in a molecule provides a valuable way to characterize a radical arising from the molecule.

Since the earlier report we have concentrated on routes to synthesize the deuterated choline chlorides. At first, attemps were made to deuterate by exchange between D_2O and trimethylamine, and between D_2O and ethanolamine. Both acid (DCl) and basic (NaOD) catalyst were tried at temperatures up to 150°C. The amount of deuteration was established by means of the nuclear magnetic resonance signal. There was no evidence for exchange of any protons on carbon atoms.

Since D_2O was initially our only source of deuterium, we investigated the routes of Hard and Pilgrim[2] and Christopher[3] to malonic acid-d_4 and acetic acid-d_4 from D_2O and tartaric acid. Their results were verified but the work was discontinued when a commercial source of acetic acid-d4 was found. The positions on the choline chloride being deuterated are described by means of a numbered model.



[1] Robert Lindblom and Richard M. Lemmon, in Chemistry Division Quarterly Report, UCRL-3629, Jan. 1957, p. 24.

[2] C. D. Hurd and F. D. Pilgrim, J. Am. Chem. Soc. 55, 757 (1933).

[3] W. L. Christopher, J. Chem. Soc. 429 (1935).

A. Deuteration of the (1) Position

This deuteration is easily accomplished by direct exchange with D_2O.

B. Deuteration of the (2) or (3) Position

Direct reduction of the methyl ester of betaine chloride, $[(CH_3)_3NCH_2CO_2CH_3]^+Cl^-$, with lithium aluminum deuteride has not been tried for several reasons: first, the betaine esters are very insoluble in the solvents used with $LiAlD_4$ (ether or tetrahydrofuran); second, there are no encouraging literature references. In addition, there is another route for which all the steps are in the literature. This route involves the rearrangement of betaine to methyl N, N-dimethyl glycine,[4] the reduction of this compound to an amino alcohol,[5] and the quaternization of the amino alcohol to choline iodide with methyl iodide. By using lithium aluminum deuteride in the reduction, one obtains the choline iodide deuterated in the (2) position. On the other hand, starting with betaine from bromoacetic acid-d_3 and trimethylamine, and reducing the product with lithium aluminum hydride, yields choline deuterated in the (3) position.

C. Deuteration of the (4) Position

The first route tried involved reaction of ammonia with methyl chloroformate to give methyl urethane, followed by reduction of the urethane with lithium aluminum hydride. Repeating the addition of methyl chloroformate and reduction for three consecutive times should give trimethylamine, which can be reacted with ethylene chlorohydrin to give choline chloride. However, the reaction failed at the first reduction step. Since the second and third reductions are successful, it is possible to prepare methylamine by means of the Hofmann reaction, $CD_3CONH_2 \xrightarrow{NaOCl} CD_3NH_2$. This bypasses that step of the first route which was unsuccessful. The failure of the first reduction is attributed to the loss of the N protons, resulting in an insoluble ionic crystal.

Another route that has been partially investigated is the use of the Hunsdiecker reaction on the silver salt of acetic acid-d_3 to get methyl-d_3 bromide. Choline bromide is then made by trimethylation of ethanol-amine with the methyl-d_3 bromide. Although the product purification may be more of a problem in this route, the starting materials are both cheaper and more available. We have found that the Hunsdiecker reaction with silver acetate should be done at refluxing temperature with small bromine additions. If a large amount of the intermediate hypohalite is allowed to accumulate, it may decarboxylate with almost explosive rapidity.

Of the methods investigated, Willstätter's rearrangement gave a 45% yield, the Hofmann reaction gave about 70% yield, and the Hunsdiecker reaction gave a 60% yield. The reduction of amino acids to amino alcohols is reported in the literature to give 80% to 90% yields, but we have not yet investigated this reaction.

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[4] R. Willstätter, Ber. deut. chem. Ges. 35, 595 (1902).

[5] V. M. Micovic and M. L. Mihailovic, Lithium Aluminum Hydride in Organic Chemistry, Serbian Academy of Sciences Monograph 1238, Belgrade, 1955.

Considerable effort was applied to the separation of the methyl amines by vapor-phase chromatography. Since this work was pertinent only to the ammonium halide irradiation work and not to the choline chloride program, it was dropped when difficulty was experienced with irreversible absorption of the amines on the substrate. James and Martin have also reported on this difficulty in chromatographing the methyl amines.[6]

[6] James, Martin, and Smith, Biochem. J. 52, 238 (1952).

THE DECARBONYLATION OF BENZOYLFORMIC ACID

Bernice Fingerman and Richard M. Lemmon

A previous report[1] described an attempt to determine an isotope effect in the thermal decarbonylation of pyruvic acid and its esters. However, no real decarbonylation isotope effect could be measured, because large amounts of CO_2 were obtained along with the CO. Recently a paper by Banholzer and Schmid[2] described the nearly quantitative decarbonylation of benzoylformic acid; only traces of CO_2 were observed. Benzoylformic acid seems, therefore, an excellent compound on which to measure the C^{12}-C^{14} isotope effect that may be involved in the reaction of decarbonylation. This report describes the steps we have taken in that direction.

Experimental Procedure

Preparation of Carboxyl-Labeled Benzoylformic Acid

A synthesis of $C_6H_5COC^{14}O_2H$ was carried out according to the procedure of Banholzer and Schmid.[2] First, labeled potassium cyanide was prepared from metallic K, $BaC^{14}O_3$, and NH_4Cl according to the method of Sixma et al.[3] The $KC^{14}N$ was converted to $Cu_2(C^{14}N)_2$ which, in turn, was reacted with benzoyl chloride to give $C_6H_5COC^{14}N$. The labeled benzoylformic acid was then prepared by hydrolysis of the nitrile with concentrated HCl. The product had a specific activity of 0.012 μC/mg.

Decarbonylation Experiments

The decarbonylation was carried out at a temperature of 40°C by the addition of 15 ml of 98% H_2SO_4 to about 1 mmole of the benzoylformic acid. The apparatus shown in Fig. 16 was used for decarbonylation. During the reaction, constant stirring was maintained (with a magnetic stirrer), and a slow stream of helium was used to sweep the evolved carbon oxides through the system. Any evolved CO_2 was condensed in the first trap [7(a)]. The gases then passed through a heated furnace where the CO was oxidized to CO_2; the CO_2 was then condensed in the second trap [7(b)]. The reaction was continued for 6 hours. The second trap was then detached from the line and connected to the apparatus shown in Fig. 17. This system was pumped to a high vacuum and the CO_2 was quantitatively transferred to the cold finger (6) by placing a Dewar flask of liquid nitrogen at this point. The volume of the system to the left of stopcock 7 was previously calibrated; consequently, after the transfer to 6, stopcock 7 was closed, the liquid nitrogen bath was removed from 6, and the amount of CO_2 was determined by the resultant deflection of the manometer 2.

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[1] H. M. Frey, in Chemistry Division Quarterly Report, UCRL-3358, April 1956.

[2] K. Banholzer and H. Schmid, Helv. Chim. Acta 39, 548 (1956).

[3] F. L. J. Sixma, H. Hendriks, K. Helle, U. Hollstein, and R. Van Ling, Rec. trav. chim. 73, 161 (1954).

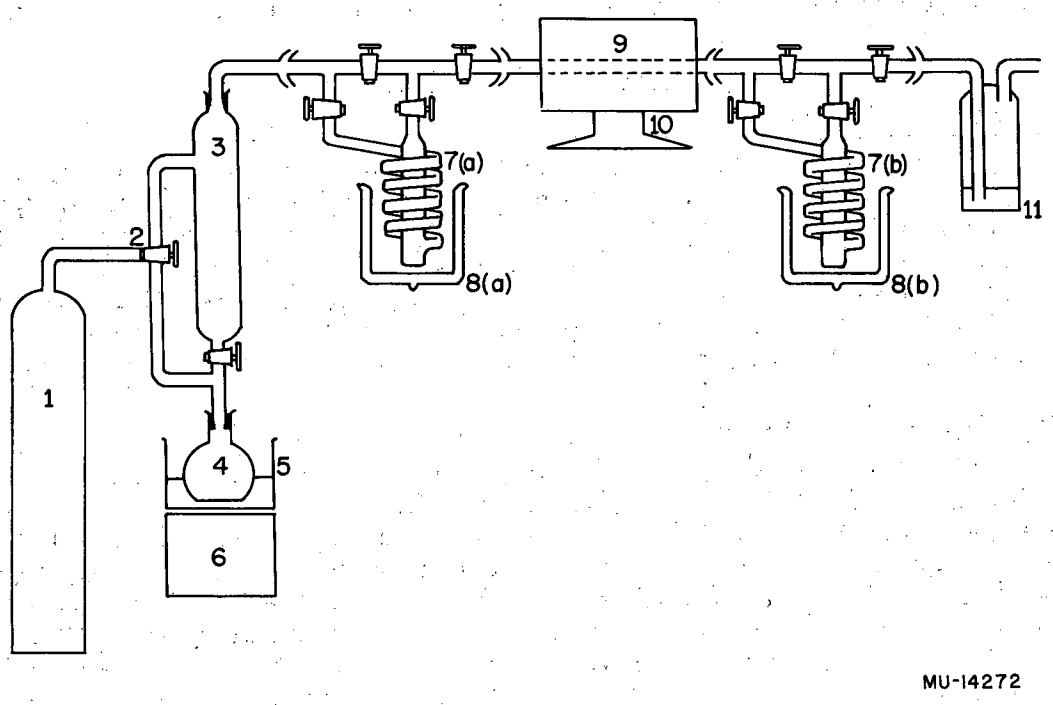

Fig. 16. Apparatus for the decarbonylation of benzoylformic acid.

1. Helium tank
2. Three-way stopcock
3. Pressure-compensated dropping funnel
4. 100-ml decarbonylation flask
5. Oil bath with heating coil
6. Magnetic stirrer
7. Spiral traps
8. Liquid nitrogen baths
9. Tube of CuO
10. Combustion furnace (610°)
11. Sulfuric acid bubbler

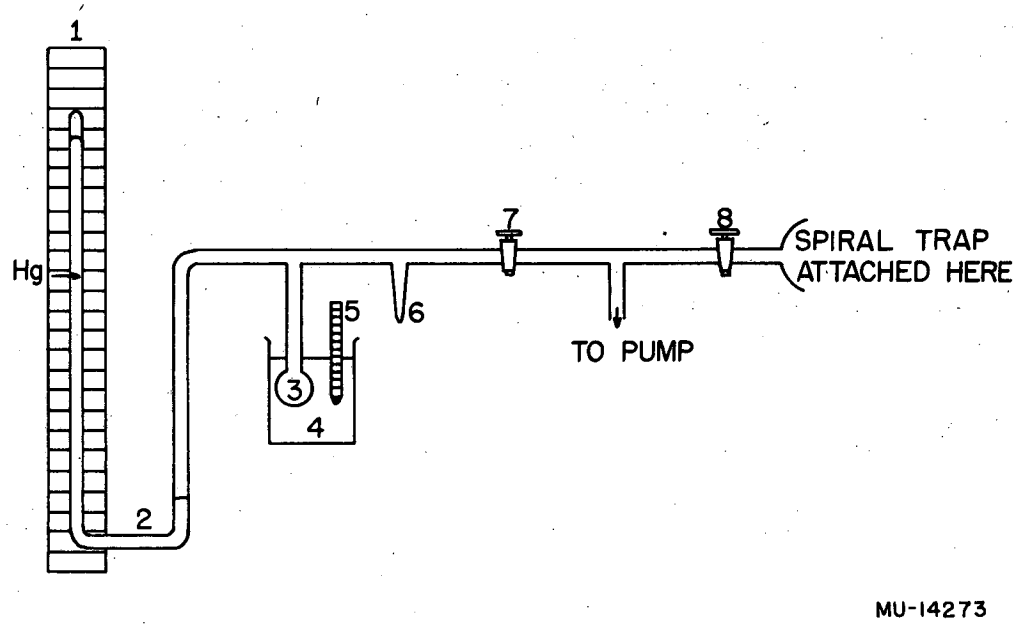

Fig. 17. Apparatus for CO_2 volume measurement

1. Meter stick
2. Mercury manometer
3. Constant-temperature bulb
4. Water bath
5. Thermometer
6. Cold finger
7, 8. Stopcocks

The samples of CO_2 were transferred into a U tube immersed in liquid nitrogen and from there into an ionization chamber by means of the vacuum line shown in Fig. 18. As a final step in this transfer, unlabeled CO_2 (up to 1 atmos. pressure) from a tank was swept into the chamber, thus flushing the system of any residual labeled CO_2. The amount of radioactivity in the CO_2 could then be determined by means of the ionization-chamber assay technique that was described earlier.[4]

About 1% of the gas evolved during the heating of the benzoylformic acid appeared as CO_2; from 90 to 99% appeared as CO. Consequently, the reaction is certainly a good one on which to measure a decarbonylation isotope effect. At the present time our data are not sufficient for us to assign a value to the magnitude of the isotope effect involved. The data so far obtained indicate a small (few percent) and positive isotope effect; that is, the C^{12}-C^{12} bonds are breaking a little more easily than the C^{12}-C^{14} bonds.

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

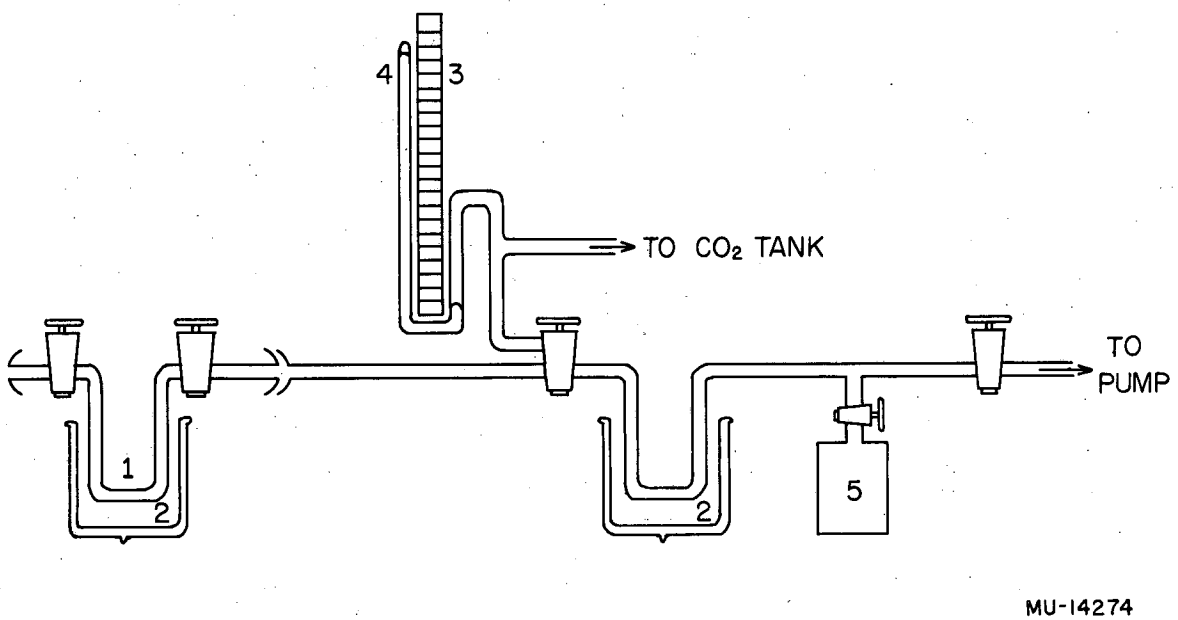

Fig. 18. Apparatus for transferring CO_2 into ionization chamber

1. U tube for transporting CO_2 sample
2. Liquid nitrogen baths
3. Meter stick
4. Mercury manometer
5. Ionization chamber

RESPIRATORY CARBON-14 PATTERNS FOR D-, L-, AND DL-NORVALINE

Martha R. Kirk, B. M. Tolbert, and Ann Ludwigsen

In a series of Quarterly Reports[1,2,3,4] we have described a method for the small-scale resolution of a number of the amino acids and presented the carbon-14 respiration patterns from rats for these acids. In this report we are presenting the carbon-14 respiration data for D-, L-, and DL-norvaline-3-C^{14}.

Experimental Procedure

Male rats of the Long-Evans strain and weighing approximately 250 grams were used in these experiments. In order to obtain a more uniform level of metabolic activity in the different animals, each was fasted for about 16 hours before the beginning of the experiment. Each was injected intraperitoneally with about 1.5 mg of the isomer to be tested, containing about 6μC C^{14} dissolved in 0.2 ml water. The animal was then placed in a metabolic cage and respiration patterns were made by the ionization chamber–continuous flow method.[5]

Results and Discussion

Figure 19 shows a plot of time vs the total percent of the injected carbon-14 that is converted to $C^{14}O_2$ in 7 hours after the injection of each of the three isomers. Each curve is the average from several animals. The specific-activity curves are presented in Fig. 20.

There is no statistical difference between the excretion as $C^{14}O_2$ of D-, L-, and DL-norvaline-3-C^{14}. This is not too surprising, since norvaline is not one of the amino acids normally obtained on protein hydrolysis and is usually ignored by the protein chemists. Little is known of the metabolic fate of this amino acid in the animal body, but the C^{14} respiration pattern would seem to indicate that the carbon moiety is excreted in much the same way as a fatty acid of comparable skeleton.

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[1] Patricia T. Adams, in Chemistry Division Quarterly Report, UCRL-3240, Dec. 1955, p. 24.

[2] Martha Kirk, Patricia T. Adams, and B. M. Tolbert, in Chemistry Division Quarterly Report, UCRL-3351, March 1956, p. 21.

[3] Patricia T. Adams, in Chemistry Division Quarterly Report, UCRL-3415, June 1956, p. 50.

[4] Martha R. Kirk and B. M. Tolbert, in Chemistry Division Quarterly Report, UCRL-3629, January 1957, p. 60.

[5] Tolbert, Kirk, and Baker, Am. J. Physiol. 185, 269, (1956).

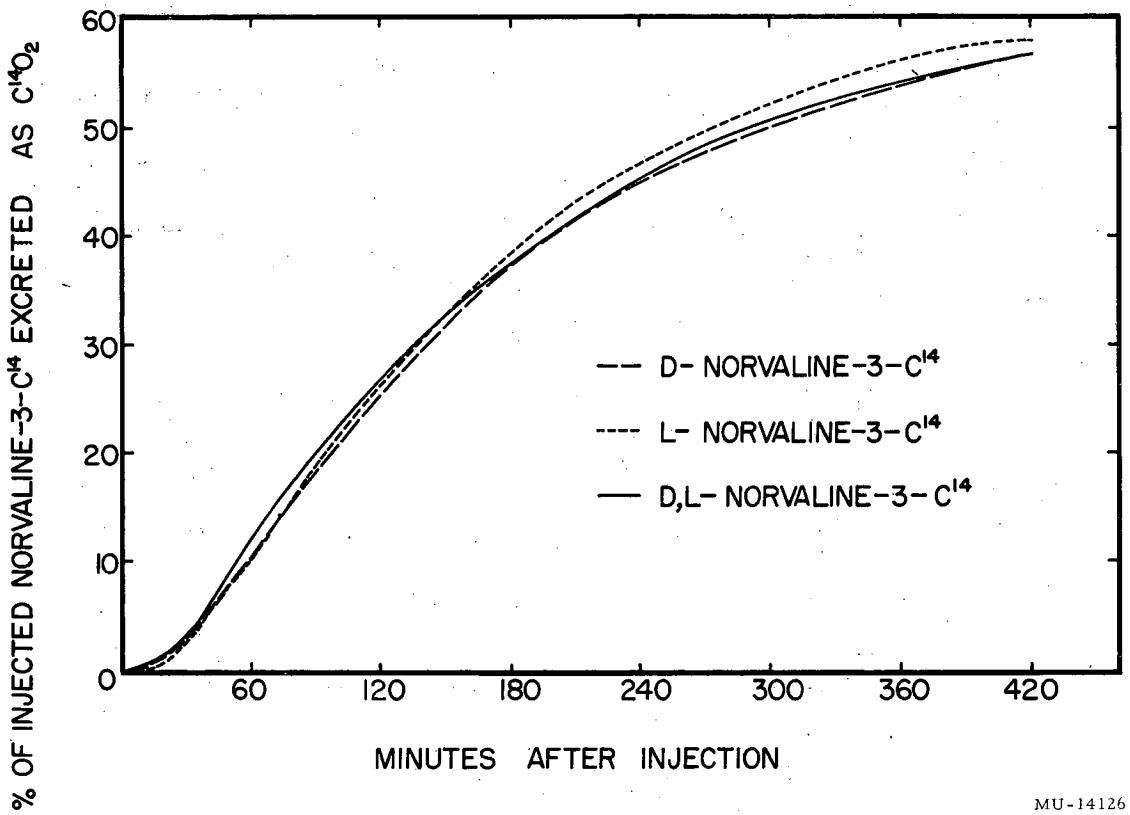

Fig. 19. Cumulative excretion of the norvaline-3-C^{14} isomers.

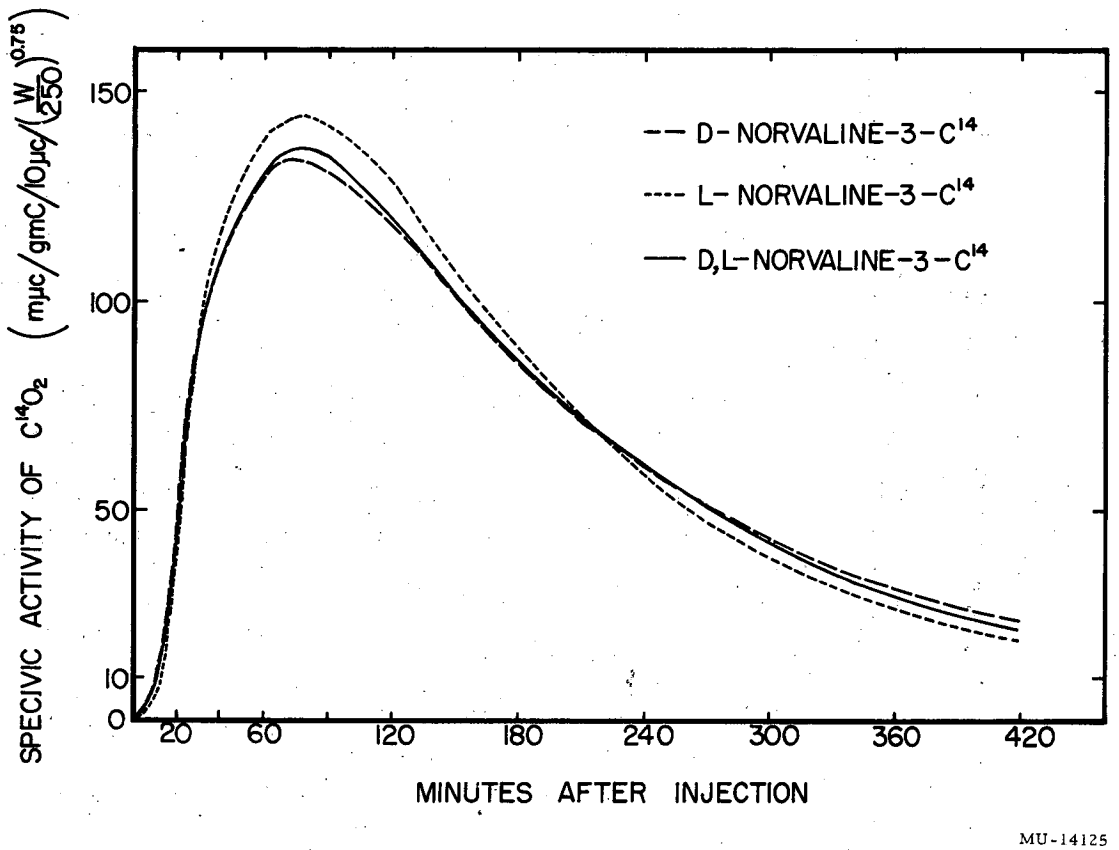

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Fig. 20. Specific activity of $C^{14}O_2$; norvaline-3-C^{14} isomers.

BIOCHEMICAL FACTORS IN LEARNING: CORTICAL CHOLINESTERASE ACTIVITY AND RATE OF LEARNING

James L. McGaugh, Edward L. Bennett, and Hilda Karlsson

(In collaboration with Lewis Petrinovich and Professors M. R. Rosenzweig and David Krech, Department of Psychology)

In previous reports we have presented evidence that, in the rat, the capacity for adaptive behavior is correlated with the level of cholinesterase (ChE) activity in the cerebral cortex. In these studies, we have used as a measure of adaptability the behavior of rats in the Krech Hypothesis Apparatus.[1] In this apparatus, rats are faced with either an unsolvable or progressively solvable discrimination problem.[2] Rats that behave more adaptively in these problems tend to have higher cholinesterase activity than rats whose responses are sterotyped or unadaptive.

The purpose of the present studies was to investigate the generality of the adaptability-cholinesterase relationship in other behavioral situations. For this purpose, we investigated the relationship between individual differences in ChE activity and individual differences in rate of learning in two situations: simple instrumental learning and maze learning.

For the simple instrumental learning problem, rats were trained to shuttle back and forth in a box 6 inches wide by 24 inches long in order to receive food rewards in one end of the box.[3] Eighteen rats from the S_{13} strain maintained by the Psychology Department were trained in this apparatus. They were first placed on a 24-hour deprivation schedule for 4 days. On the fifth day and on every subsequent day for an additional 13 days each rat was placed in the apparatus for 10 minutes, during which each shuttle was rewarded with about 1/10 g of wet mash. The number of responses made was automatically recorded on a counter. On the nineteenth day, the rats were allowed access to wet mash for 20 minutes in a feeding cage. The amount of food eaten during this period was estimated by weighing the animals before and after eating. The animals were sacrificed and analyzed for cortical ChE activity according to the procedure previously described.[4] For the 18 animals, the correlation between total number of shuttle responses over the 14-day period and the ChE activity was 0.00. The correlation between total number of responses and amount of food eaten during the 20-minute free-access period on Day 19 was + 0.90. Thus, it appears that the rate of response in this apparatus is primarily a measure of food motivation. Further, these results provide evidence that cholinesterase activity is not related to food motivation. Since all the studies of adaptive behavior and cholinesterase activity to date have used food motivation, this is an important finding. In summary, this study has shown that cholinesterase activity is not correlated with response rate in a simple instrumental learning problem.

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[1] E. L. Bennett and R. Dean, in Chemistry Div. Quarterly Report, UCRL-3415, June 1956, p. 21.

[2] Krech, Rosenzweig, and Bennett, J. Comp. and Physiol. Psych. 49, 261 (1956).

[3] J. L. McGaugh and L. Petrinovich, Am. J. Psych. 69, 660 (1956).

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

For the first maze-learning study, 35 rats were trained in the 14-unit automatically recording maze described previously.[5] The rats were first placed on a 24-hour deprivation schedule and pretrained in a straight runway for 8 days. The animals were then given one trial a day in the maze for 14 days, and errors were recorded. All animals were then sacrificed and analyzed for cortical ChE activity. Nine of the 35 animals were hyperexcitable and showed no signs of learning over the 14 trial days. (The emotionality of this strain has not been restricted to this apparatus, and has been noted in other testing procedures with this strain). For the 26 "learners," the correlation between initial errors made in learning the maze and ChE activity was + 0.47, and for the 9 "nonlearners," it was 0.00. A correlation of + 0.47 is significant at the 0.02 level of confidence. In other words, in this maze high cholinesterase activity is associated with poor maze learning.

For the second maze-learning study, animals were trained in a 4-unit 8-cul Lashley III alley maze. Thirteen animals of the S_{13} strain were first pretrained in a straight runway for 8 days and then given one trial a day in the maze for 8 days. Subsequently cortical ChE activity was determined. For the 13 animals, the rank-order correlation between initial errors and ChE activity was + 0.28.

Four other groups of animals were also run in the Lashley maze, but following a somewhat different schedule. They were given one trial on Day 1, two trails on Day 2, two on Day 3, and five trials per day on Days 4 through 7. The following results were obtained:

Strain	N	Mean initial errors	Correlation, cortical ChE vs errors
S_1	13	19.2	0.53
S_3	14	42.3	0.42
CH	11	38.8	0.39
CL	11	28.1	- 0.06

(The S_1 and S_3 rats were those used to study the effects of monosodium diet, described in another section of this quarterly report, p. 91).

The correlations are not significant, with the small number of animals used in each group. Nevertheless, it should be noted that three of the four correlations in the table are positive, and the correlation with the S_{13} animals was also positive. These findings suggest that in the Lashley III maze, cortical ChE activity correlates positively with the number of errors made during learning; that is, ChE activity correlates negatively with the rate of learning the maze.

[5] J. L. McGaugh and E. L. Bennett, in Chemistry Division Quarterly Report, UCRL-3629, Jan. 1957, p. 48.

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If the learning of these mazes is taken as a measure of adaptiveness, these results are perhaps not what might have been expected on the basis of our previous studies using the Krech Hypothesis Apparatus. In the Hypothesis Apparatus, the basic measure of adaptiveness has been the degree to which the animals give up an incorrect solution (or "Hypothesis") in an unsolvable situation. In the maze, the measure recorded is the number of errors made in a situation where a specific spatial pathway must be learned. The two behavioral measures are quite different, and it appears that they are not related in the same way to cortical ChE activity.

These results are not considered as contradicting our earlier findings that ChE is positively correlated with adaptive behavior as measured in the Hypothesis Apparatus. They do suggest, however, that further studies of behavioral correlates of ChE activity will prove fruitful.

GENETIC INVESTIGATIONS OF CHOLINESTERASE ACTIVITY IN THE CEREBRAL CORTEX OF THE RAT

Thomas H. Roderick, Edward L. Bennett, and Hilda Karlsson

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

In conjunction with the study of brain chemistry and behavior initiated at Berkeley by Drs. D. Krech, M. R. Rosenzweig, and E. L. Bennett, a genetic program has been undertaken. This program, which is primarily concerned with the cholinesterase (ChE) activity of specific areas of the rat cerebral cortex, has two purposes: (a) to help to elucidate the genetic mechanism involved in the inheritance of this enzyme, and (b) to establish high- and low-ChE strains of rats with subsequent study and search for possible concomitantly exaggerated behavior patterns, neurohistological differences and associated neurochemical changes.

A selective breeding program has now been carried through the fourth generation. At the outset of the program it was decided to use two foundation stocks from which selected lines would be derived. Thus, selecting while keeping the stocks completely separate would afford a simultaneous replication of the selection experiment. The stocks available were dissimilar in their genetic backgrounds, but similar in that they were both genetically highly heterogeneous--a prerequisite for selection success. Stock 1, the Castle stock, was obtained from the rat colony of Dr. W. E. Castle, Department of Genetics, University of California. These animals were descendants, through several generations of random mating, of many different lines. Stock 2, the Dempster stock, was obtained from a four-way cross of four unrelated inbred lines maintained in the Genetics Department Rat Colony in Berkeley. Both stocks, therefore, represent a sample of a hypothetical population of rats.

From each of these stocks, one high- and one low-ChE line have been selected, using a double-first-cousin mating system. This system is broad enough to maintain a sufficient amount of genetic variability upon which to select over several generations, and yet close enough that our rat population has not exceeded 500 animals. The selection criterion is the highest or lowest average ChE activity in the visual and somesthetic areas (v + s)/2 of the cerebral cortex.[1] The ChE activity is assayed only in the males. Two litters from each line are used. The males of one litter are crossed with the females of the other, and vice versa. After having produced offspring, the males (sires) are sacrificed and chemically analyzed. The offspring from the males with the highest (v + s)/2 ChE value (in the case of a selected high line) in each litter are saved, thus offering two litters of the next generation upon which the process is repeated (Fig. 21). The procedure for low-ChE selection is similar except that offspring of the males with the lowest ChE values are saved.

Figure 22 and Table XXIII summarize the results of selection to date. Whenever possible, the high and low lines within each stock are chemically analyzed together, making the difference between the means a more valuable statistic. An associated shift of the Castle lines is observed between generations, whereas no such shift has been seen in the Dempster lines. This shift may be caused by possible seasonal effects. Further investigation into the magnitude and origin of this and other environmental effects is now in progress.

Although the means of the high and low lines differ significantly ($p < 0.01$ for S4, inferring from the stocks combined or taken separately), the response has been asymmetrical. The Castle high line shows no response to selection, and the Dempster high line shows very little response compared with the low lines. Natural selection, possibly through epistatic effects, may be holding the high lines at a plateau, although there is no correlation between the extreme highs or lows and lowered fecundity. To shed some light on this problem, high-by-high and low-by-low crosses between the stocks have been made. Increased genetic variability may allow further selection response in the high direction, and by the use of such crosses, the amount of similarity between the genes or gene complexes for high and low ChE activity between the two stocks can be better understood.

Along with the selection program, several inbred lines derived from the Genetics Department Rat Colony are being analyzed as well as reciprocal crosses between them. Through these experiments, the magnitude of possible maternal and heterotic effects may be estimated. Also from the selected lines, animals are being raised which will be subjected to a variety of behavior tests.

[1] Krech, Rosenzweig, Bennett, and Krueckel, Science 120, 3128 (1954).

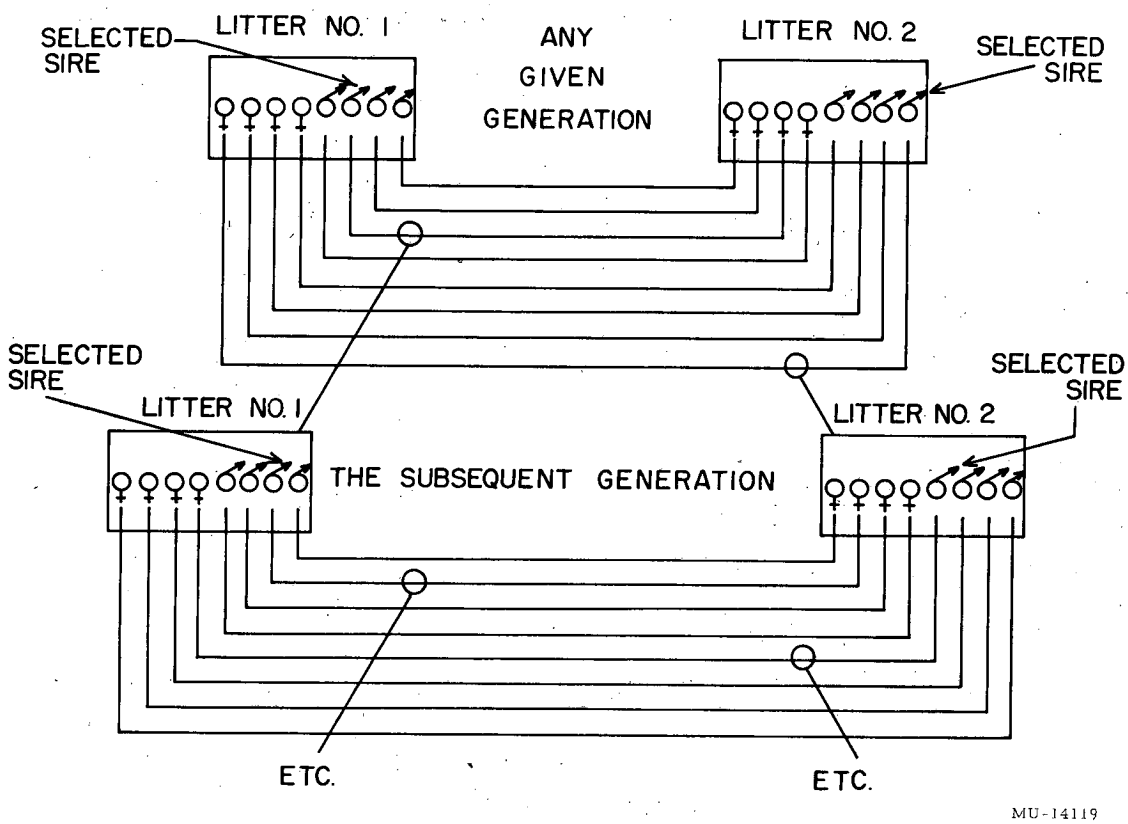

Fig. 21. The double-first-cousin mating scheme employed in each line, showing how selection is made.

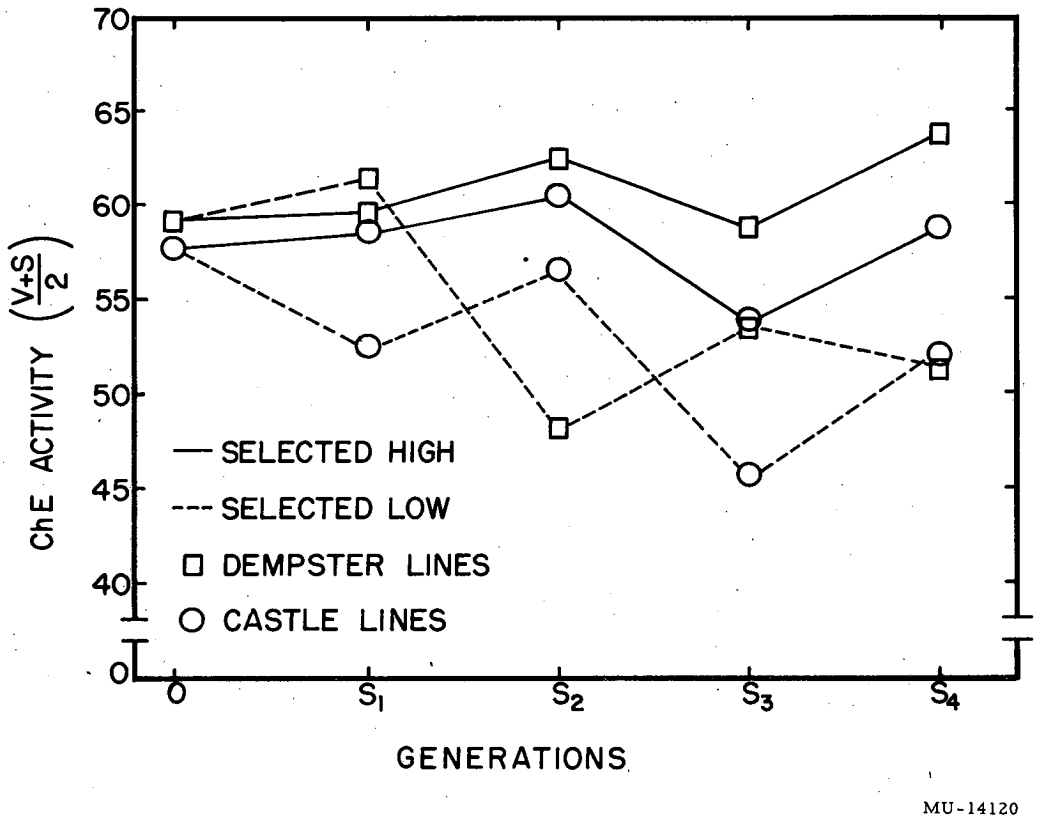

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Fig. 22. Results of selection for high and low ChE activity of the cerebral cortex in the rat.

Table XXIII

Selection results. ChE values (v + s)/2 of foundation stocks and four generations (moles of ACh x 10^{10} hydrolyzed per minute per mg of tissue)

Generations	Foundation Stock					
	Dempster			Castle		
	N 14 $\bar{x}$[a] 59.05 σ 4.52			N 14 $\bar{x}$ 57.70 σ 6.56		
	High	Low	High minus Low	High	Low	High minus Low
S1	N 8 $\bar{x}$ 59.66 σ 3.54	7 61.41 5.42	-1.75	4 58.38 2.39	9 52.10 4.42	6.28
S2	N 9 $\bar{x}$ 62.31 σ 3.27	8 48.00 3.03	14.31	8 60.44 5.63	6 56.45 5.55	3.99
S3	N 10 $\bar{x}$ 58.77 σ 4.29	7 53.26 2.42	5.51	9 53.30 2.61	6 45.37 3.93	7.93
S4	N 10 $\bar{x}$ 63.58 σ 3.45	8 51.11 2.13	12.47	9 58.81 3.31	8 51.82 2.69	6.99

[a]The term $\bar{x}$ stands for the mean value for cholinesterase.

MONOSODIUM GLUTAMATE DIET AND MAZE PERFORMANCE

Edward L. Bennett and Hilda Karlsson

(In collaboration with
Professors M. R. Rosenzweig and David Krech,
Department of Psychology)

There has long been a controversy as to whether glutamic acid or monosodium glutamate (MSG), as dietary supplements, can improve intellectual performance. Recently Hughes and Zubek[1] have concluded "that glutamic acid can increase the learning ability of dull rats considerably. It does not, on the other hand, affect the learning of bright rats." They suggest that the previous negative results have been found with bright subjects--human and animal--and the previous positive results with dull subjects. They hypothesize that monosodium glutamate may exert a beneficial effect by aiding synthesis of acetylcholine, thus favoring neural transmission.

We have attempted to replicate their work, using rats of our S_1 strain (descendants of the Tryon Bright strain) and rats of the S_3 strain (descendants of the Tryon Dulls). Within each strain, littermates were divided into two groups. Half were given a daily dietary supplement of monosodium glutamate, following the procedure used by Hughes and Zubek. The supplement was given from weaning, at about 25 days of age, until testing, at about 105 days. For each strain, seven pairs of littermates were used for the behavioral test, one littermate having been fed the MSG supplement and the other the control diet.

In order to test the conclusion reached by Hughes and Zubek, it is necessary to use a behavioral situation in which the performance of one strain is clearly superior to that of the other strain. A preliminary experiment had shown that animals of the S_3 strain make about twice as many errors in the Lashley III maze as do animals of the S_1 strain. It was also shown that the reliability of scores in this maze is satisfactory. The results are presented in Table XXIV.

It is clear that the MSG diet did not improve performance; in fact, in 9 out of the 14 pairs of littermates, the MSG-fed animal made more errors than his control. The strains, however, were significantly different; the S_1 rats made less than half as many errors as the S_3 rats.

These results indicate that the MSG supplement is not beneficial to all dull strains of rats. It may be that the dull strains of Hughes and Zubek has a special neurological deficiency that can be remedied by the MSG diet. Specific, genetically determined "biochemical lesions" have been found in other cases, and it might be worth while to attempt to characterize the chemical defect of their dull strain. At the same time, it is clear that a behavioral trait--especially one of a general nature such as "dullness"--may arise from any of a large number of causes.

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[1] K. R. Hughes and J. P. Zubek, Can. J. Psychol. 10, 132 (1956).

Table XXIV

Initial errors in Lashley III maze
($\bar{x}$ = av. no. of errors made; σ = standard deviation)

Strain	S_1		S_3	
Diet	Control	MSG	Control	MSG
No. of animals	7	7	7	7
$\bar{x}$	16.9	20.7	41.1	43.4
σ	5.6	7.2	21.5	15.5

(These animals are the S_1 and S_3 rats whose data are also presented in the article, "Biochemical Factors in Learning," p. 84, in this Quarterly Report.)

BRAIN LACTIC DEHYDROGENASE ACTIVITY OF RATS SELECTED FOR DIFFERENCES IN BRAIN CHOLINESTERASE ACTIVITY

Hilda Karlsson and Edward L. Bennett

(In collaboration with
Professors David Krech and Mark Rosenzweig,
Department of Psychology)

Part of our program of relating adaptive behavior to brain chemistry involves the selective breeding of animals for differences in brain cholinesterase activity. The selective breeding has brought about significant differences in ChE activity.[1] We have now tested to determine whether these differences are specific to the enzyme upon which selection is based, or whether more general differences are being produced. It is possible, for example, that the differences in ChE activity reflect only widespread differences in the level of brain metabolism, and that the selection is operating directly on brain metabolism and only secondarily on ChE activity.

To test the specificity of the effects of selection, we have determined the activity of the enzyme lactic dehydrogenase (LD) in the brains of animals selected for high and for low cortical ChE activity.[2] (Lactic dehydrogenase reduces pyruvic acid to L-lactic acid and acts as a shunt from the tricarboxylic acid cycle. LD provides an anaerobic route for the oxidation of glucose in the brain, and prevents the buildup of pyruvic acid.) The animals considered are the high-ChE strain and the low-ChE strain being developed from the heterogeneous Castle Stock.[1] The particular animals used in this test were largely collaterals of the third selected generation of the Castle High ChE and the Castle Low ChE strains. Animals of both strains were sacrificed for chemical analysis at about 115 days of age; only male animals were used. Tables XXV and XXVI present data for only those animals for which both ChE and LD activity were determined. For each animal separate analyses were made from samples of the visual area of the cerebral cortex (V), the somesthetic area of the cortex (S), and the brain remaining after all cortex had been stripped off (Br). Since the LD values may not be completely comparable from one day of analysis to another, values are entered separately for each day of analysis.

When the ChE values of the two strains are compared, section by section, in Table XXV, it is seen that the Castle Low strain has less ChE activity than the Castle High strain. (The only exception is found in the section in which only two Castle Low animals are represented.) Over-all, the Castle Low strain has about 95% as much ChE activity as the Castle High strain. With larger groups, we have found the difference in cortical ChE activity between these strains to be significant.

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[1] T. R. Roderick, E. L. Bennett, and Hilda Karlsson, in this quarterly report, p. 86.

[2] A spectrophotometric assay based upon the rate of reduction of DPN under standardized conditions in the presence of lactic acid was used. The details will be described in a later report.

When the LD values are compared in Table XXVI, it is seen that, contrary to the case in Table XXV, the Castle Low strain has higher values than the Castle High strain. The differences average about 2% for the cortical analyses; there is no difference in the subcortical brain. It is not possible with the present data to calculate the significance of the observed differences.

Since the differences between the strains are in opposite directions for the two enzymes, it is clear at least that the data do not support the hypothesis that a general factor (such as level of brain metabolism) accounts for both ChE and LD values. Furthermore, the data for LD and ChE across individual animals do not suggest that the values of the two enzymes are correlated within either strain. Pending further investigation, we conclude that the values of the two enzymes are independent.

Table XXV

Cholinesterase activity, mean comparative values

Castle Low strain				Castle High Strain			
No. of animals	Visual cortex	Spatial cortex	Brain	No. of animals	Visual cortex	Spatial cortex	Brain
6	52.1	57.2	135.3	5	55.7	59.6	141.6
3	51.8			3	58.0		
2		64.1		4		62.0	
7		56.8		4		61.6	
Av.	51.9	57.9	135.3	Av.	56.6	60.9	141.6

Table XXVI

Lactic dehydrogenase activity, mean comparative values

Castle Low Strain				Castle High Strain			
No. of animals	Visual cortex	Spatial cortex	Brain	No. of animals	Visual cortex	Spatial cortex	Brain
6	156.9	158.0	128.0	5	148.6	153.3	127.7
3	148.9			3	153.9		
2		157.8		4		155.6	
7		148.7		4		146.2	
Av	154.2	153.6	128.0	Av	150.6	151.8	127.7

PRELIMINARY EXPERIMENTS ON HEXOKINASE ACTIVITY IN RAT BRAIN

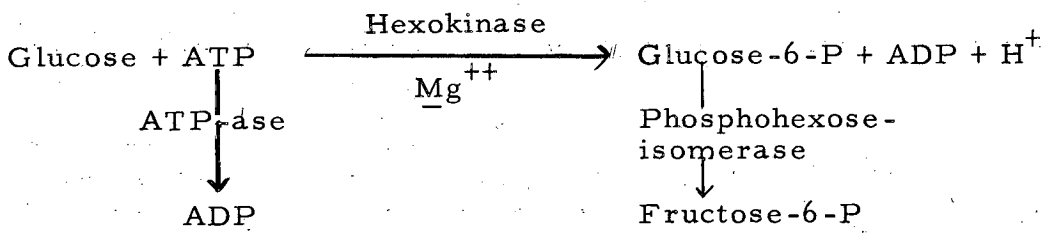
Ann E. Ohlander and Edward L. Bennett

(In cooperation with
Professors Mark R. Rosenzweig and David Krech, Dept. of Psychology)

As a continuation of the investigations relating brain metabolism with behavioral patterns in rats,[1,2] a series of preliminary experiments for determining the level of hexokinase activity was begun.

Hexokinase was chosen because it can be the limiting factor in the cerebral glycolytic cycle, as it has the lowest activity rate of all the individual glycolytic enzymes thus far determined (390 μmoles per gram of fresh weight of brain per hour, compared with the second lowest value of 500 μmoles).[3] This cycle provides the energy by which choline is acetylated in or around the neural synapses and thereby indirectly controls conduction. The usual rate of neural activity is not great enough to require maximum energy, and therefore hexokinase acts only as a control when the brain undergoes unusual stimuli.[4]

The specific chemical reaction involved is the phosphorylation of hexose to hexose-6-phosphate by removal of a phosphate group from adenosine triphosphate (ATP). As a nonpurified homogenate would be used, certain side reactions had to be considered. The two most important ones were due to the presence of adenosine triphosphatase (ATP-ase), which breaks down ATP to its diphosphate (ADP), and the presence of phosphohexoseisomerase, which causes the conversion of glucose-6-phosphate to fructose-6-phosphate.



One first step was to find a suitable method by which the rate of reaction could be quantitatively determined in a nonpurified homogenate. Several methods have been considered: (a) disappearance of the substrate, (b) formation of product, i.e., hexose-6-phosphate, (c) formation of acid, and (d) disappearance of thermolabile phosphate groups. We found the last two methods impractical for us because of the action of ATP-ase in the

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

[2] Bennett, Krueckel, and Halevy, in Chemistry Division Quarterly Report, UCRL-3157, June 1955, p. 28.

[3] Henry McIlwain, Biochemistry and the Central Nervous System (Little, Brown and Company, Boston, 1955) p. 67.

[4] Ibid, p. 69.

crude homogenate, therefore procedures based upon the disappearance of substrate or of glucose or upon the formation of hexose-6-phosphate have been investigated. In each case preliminary-model experiments have been carried out using yeast hexokinase (Sigma type III) to test the procedure.

One attempt to quantitate hexokinase activity was by the incubation of glucose-C^{14} with yeast hexokinase or rat-brain homogenate, and subsequent separation of the glucose and hexose-6-phosphates (glucose-6-P and fructose-6-P) by paper chromatography. A standard incubation mixture was used containing the following concentrations: glucose, 0.0012M; ATP, 0.005M; $MgCl_2$, 0.005M; KF, 0.05M; KCl, 0.042M; K_2HPO_4-KH_2PO_4 buffer, 0.1M; final pH for the entire system, 7.1 to 7.3. At zero time 50 μg yeast hexokinase was added to the standard reaction mixture, which also included approximately 0.3 μC of glucose-C^{14} (~300 μC/mg). The mixture was placed in a 37°C water bath, and at times varying from zero to 15 minutes aliquots were removed and mixed with 10% trichloroacetic acid to precipitate the protein. Portions of the supernatant were then placed on unwashed Whatman No. 1 filter paper. These papers were chromatographed first in phenol-water solvent for 24 hours and then in butanol–propionic acid–water for another 24 hours. Autoradiographs made of these chromatograms located the radioactive compounds, and the ratios of activity in the glucose and hexose-6-phosphate areas were determined by counting with a Scott tube. This procedure not only produced an easily quantitated linear relationship when yeast hexokinase was used, but also provided a means by which we could determine what other compounds might possibly be formed and interfere with measurement of hexokinase activity.

When this procedure was applied to rat-brain homogenate (brain without cortex ground in cold saline), the rate of activity fell far below the expected value (~4.0 μM glucose phosphorylated per gram of brain per hour instead of ~400 μM glucose phosphorylated). In comparing our results with those of Long et al.,[5] we found that in his procedure the tissue had been homogenized in a phosphate-fluoride buffer pH 7.8 (K_2HPO_4-KH_2PO_4, 0.1M; KF, 0.15M). Consequently, we made analyses for hexokinase activity using homogenates prepared in phosphate-fluoride, 0.9% saline, and water. The glucose remaining after incubation was determined by the Somogyi-Nelson colorimetric procedure.[6] (Preliminary experiments to standardize this procedure had been carried out previously with yeast hexokinase). Both the water and saline homogenates showed greatly reduced hexokinase activity as compared with the phosphate-fluoride homogenate. Values comparable to those reported by Long were obtained when the rat brains were homogenized directly in the phosphate-fluoride buffer. Apparently, when brain tissue is homogenized in water or saline, hexokinase is rapidly inactivated; neither the addition of KF to the water or saline homogenates nor readjustment of the pH of the homogenate to 7.8 restored the hexokinase activity to normal. The incubation procedure described above is applicable for approximately

[5] C. Long, Biochem. J. 50, 407 (1952).

[6] N. Nelson, J. Biol. Chem. 153, 375 (1944).

3 mg of brain or cortex with a 10-minute incubation period. The rate of activity can be assayed by determining the glucose remaining (Somogyi-Nelson procedure), or by determining the ratio of glucose-C^{14} to hexose-C^{14}-6-phosphate after chromatographic separation.

Another method we have tried involves the determination of the glucose-6-phosphate formed by means of the action of Zwischenferment (hexosemonophosphate dehydrogenase) and triphosphopyridine nucleotide (TPN).

$$\text{glucose-6-P} + \text{TPN} \xrightarrow{\text{Zwischenferment}} \text{phosphogluconic acid} + \text{TPN}\cdot\text{H}$$

The rate of reduction of TPN can be measured in the spectrophotometer at 340 mμ and is directly proportional to the activity of hexokinase, provided that the rest of the enzymes are present in excess. Unfortunately, the preparation of Zwischenferment also had high hexokinase activity, and therefore unless a more purified preparation can be obtained, this method must be abandoned.

At the present time, a reinvestigation of the reaction products obtained from glucose-C^{14} with the considerably more active brain or cortex homogenates has been started. Further experiments are planned to reduce sources of error, increase reproducibility and improve sensitivity of both methods. It is hoped that one of these procedures will eventually evolve into a suitable form for furthering the studies of the correlation of behavioral patterns in rats and brain metabolism.

NUCLEAR CHEMISTRY

Glenn T. Seaborg and Isadore Perlman in charge

PRODUCTION OF MICROGRAM QUANTITIES OF ACTINIDE METALS

Burris B. Cunningham and James C. Wallmann

Recently many experiments have been carried out in an effort to improve the technique of producing microgram or submicrogram quantities of the actinide metals. The reaction utilized to produce the metals is the reduction of the actinide trifluoride with metallic barium. A single globule of actinide metal surrounded by barium fluoride slag should result from the proper conditions of reduction. The variables studied include:

(a) the method of heating the reduction crucible,
(b) the rate of effusion of the barium metal,
(c) the temperature at which the reduction is carried out,
(d) the effect of various crucible materials to contain the product, and
(e) the physical form and treatment of the actinide trifluoride.

Careful control of all these factors has been found necessary when only microgram quantities of metal are to be produced and handled.

Further work is being carried on to characterize the trifluoride starting materials; the form and composition of these trifluorides have proven to be important variables in the reduction technique.

BETA DECAY OF NEPTUNIUM-238

Royal G. Albridge, Jr., and Jack M. Hollander

The conversion-electron spectrum following beta decay of Np^{238} has been re-investigated with high-resolution permanent-magnet electron spectrographs. Several new transitions are observed and assigned as arising from states of the beta and gamma vibrational bands. Comparison is made of the experimental branching ratios of transitions from each level to the various members of the ground-state rotational band with the theoretical ratios expected from the Bohr-Mottelson model, and fair agreement is obtained in the cases of known electric quadrupole transitions. In several cases of disagreement it is postulated that electric monopole transitions are being observed.

HEAVY ISOTOPES OF GADOLINIUM AND TERBIUM

Kenneth T. Faler and Jack M. Hollander

The investigation of heavy isotopes of gadolinium and terbium is being continued. From neutron capture by separated Gd^{160} we have produced an activity of several years' half-life tentatively assigned as Gd^{162}. We have been unsuccessful in attempts to separate from this activity a 14-minute terbium daughter which had been assigned as Tb^{162}, and we conclude that this activity is either incorrectly assigned or is not produced by the beta decay of Gd^{162}. Cyclotron bombardments of Gd^{160} with alpha particles have produced in the terbium fractions isotopes of half-lives of 2 and 8 hours, and preliminary excitation-function experiments indicate that they are Tb^{162} and Tb^{163}. Neither activity appears, however, to be the daughter of Gd^{162}.

NUCLEAR REACTION MECHANISMS

Bernard G. Harvey and William H. Wade

The study of nuclear reaction mechanisms in heavy elements is complicated by the high probability of fission. For example, spectra of emitted neutrons contain far more fission neutrons than neutrons from such reactions as (α, xn). It seems possible that some information about the energies and angular distributions of emitted particles can be obtained from a study of the recoiling nuclei from which the particles came.

The angular distribution of At^{211} nuclei recoiling from the reaction $Bi^{209}(\alpha, 2n)At^{211}$ has been studied experimentally. The distributions show interesting variations with the helium ion energy.

Preliminary results from Monte Carlo calculations show that the experimental results may be fitted accurately by a simple evaporation model for helium ion energies up to about 35 Mev. At helium ion energies higher than this, the experimental distributions are narrower than the evaporation model predicts. It seems likely that the high-energy experimental results will require a model in which one neutron is emitted with high energy in the forward direction, while the second neutron is evaporated with lower energy.

EXCITATION FUNCTIONS FOR THE REACTIONS $Ni^{58}(\alpha, \alpha p)Co^{57}$, $Ni^{58}(\alpha, \alpha pn+\alpha, \alpha d)Co^{56}$, AND $Ni^{60}(\alpha, \alpha pn+\alpha, \alpha d)Co^{58}$

Herbert M. Blann and T. Darrah Thomas

Stacked nickel foils have been bombarded and the cobalt isotopes produced by the (α, αp), (α, αpn), and (α, αd) reactions have been isolated chemically. The disintegration rates of the cobalt isotopes have been measured by gamma-pulse analysis. Results for various energies are given below (in millibarns).

Bombarding energy (Mev)	Reaction: $Ni^{58}(\alpha, \alpha p)$ (mb)	$Ni^{58}(\alpha, \alpha pn)$ $(\alpha, \alpha d)$ (mb)	$Ni^{60}(\alpha, \alpha pn)$ $(\alpha, \alpha d)$ (mb)
46.0	132	176	218
46.0	105	163	239
42.8	123	81.4	119
40.6	156	46.8	80
39.3	213	33.7	60
37.2	185	9.2	43
35.8	230	3.82	16.5
34.0	240	1.2	12.8
29.2	159	~ 0	7.6
26.8	110	0.12	7.4
24.3	45.4	~ 0	7.0
22.0	5.1	~ 0	5.7
17.4	0.67	~ 0	4.8
0	0.07	~ 0	1.2
0-	0.06	~ 0	

Errors are approximately ± 10% probable.

MASS SPECTROSCOPY: POLYMERS OF POTASSIUM

Frederick L. Reynolds and Maynard C. Michel

The presence of potassium as polymers K_2, K_3, etc., from hot tungsten filaments has been noted by the mass spectrometer groups both here at Berkeley and at the Livermore Laboratory for some time. Our main interest was in reducing the amount of potassium impurity in commercial pure tungsten, because intense potassium-ion beams cause secondary electrons to be formed in the ion source. This condition leads to energy spread of the sample beam. In addition, polymer formation as outlined below gives mass intensities at often unwanted masses, making high-sensitivity mass analysis a distinct problem.

This potassium impurity probably is in the parts-per-million range in commercial tungsten. It could not be detected with optical emission spectroscopy methods, yet we can see positive-ion potassium beams of the order of 10^{-10} ampere for as long as the filament lasts, the filament temperature being about 2500°K.

Tungsten, tantalum, and rhenium give potassium polymers at high temperature. No attempt has been made at the present to intercompare their intensity ratios. However, one run was made on a tantalum filament, in which the change of K_1, K_2 and K_3 with time was recorded, with the filament operating at about 2100°C. The following table shows the ratio changes with running time.

Table I

Ratio changes in K_1, K_2, and K_3 with time (Ta filament)

Time (hr)	K_1/K_2	K_1/K_3	K_2/K_3
0	4.6×10^3	7×10^3	1.66
1.5	2.1×10^3	3.3×10^3	1.57
3.5	2.9×10^3	2.4×10^3	1.40
4.0	1.5×10^3	2.5×10^3	1.66
6.0	1.7×10^3	2.0×10^3	1.15
6.5	1.3×10^3	1.5×10^3	1.16
8.0	8.4×10^3	9.8×10^2	1.16
9.0	7.2×10^2	7.2×10^2	1.06
11.0	4.1×10^2	4.3×10^2	1.06
12.0	3.5×10^2	3.2×10^2	0.92
13.0	2.5×10^2	2.5×10^2	1.00
13.4	2.0×10^2	Filament burned out	

Seaborg, Perlman

It is interesting to note that the ratio of K_2(mass 78) to K_3(mass 117) remained essentially constant. With time the actual intensity of the polymers increased relative to the monomer K_1. This is also true when tungsten or rhenium is employed as filament. In one rhenium filament the ratio of K_1/K_2 near the burn-out point was approximately 100, and with tungsten it was 70.

After a tungsten filament had been run at 2500°K for several hours the following relative intensities of the potassium polymers was recorded. All spectra were collected on a 12-inch-radius mass spectrometer, using a sixteen-stage electron multiplier with an approximate gain of 10^6 to 10^7, but no attempt is made to quote absolute beam intensities.

Table II

Relative beam intensities of potassium polymers from a W filament

Mass	Species	Relative beam intensity	Ratio K_1/polymer
39	K_1	7×10^6	---
78	K_2	1.5×10^5	47
117	K_3	1.0×10^5	70
156	K_4	7×10^3	1000
195	K_5	7×10^3	1000
234	K_6	7.5×10^2	10^4
273	K_7	8.4×10^1	10^5
312	K_8	10	7×10^5
351	K_9	22	3.5×10^5
390	K_{10}	4	2×10^6
429	K_{11}	16	4.3×10^5
468	K_{12}	9	8×10^5
507	K_{13}	9	8×10^5
546	K_{14}	6	1.2×10^6
585	K_{15}	6	1.2×10^6

We have become sufficiently interested in this problem to ascertain what the polymer intensity may be in the vapor state above the solid or molten potassium metal. Experiments on this phase have not been done to date. The formation of potassium polymers within the grain-boundary structure of a hot metal may be unique in itself and may be a function of both the pressure and the temperature of the impurity potassium in the hot filament.

SPECTROCHEMICAL DETERMINATION OF THE STRONTIUM-TO-CALCIUM RATIO IN THE FOOD, BLOOD, MILK, FECES, AND URINE OF COWS

George V. Shalimoff, Ann E. Pitzer, and John G. Conway

A dc carbon arc method has been developed for the determination of Sr-to-Ca ratios. Actual samples of milk, cream, and blood and ashed samples of food, feces, and urine are excited at 3 amperes for one minute on 1/4-inch electrodes. The wave lengths of the lines used for the working curves (log intensity Sr/Ca versus log concentration Sr/Ca) are Sr, 4607.3 Å; Ca, 4585.9 Å and 4299.0 Å. The products of several cows have been analyzed. Typical results for one cow* are:

	Sr/Ca
Skim milk	7.8×10^{-4}
Serum	5.9×10^{-3}
Cream	1.0×10^{-4}
Feces	1.1×10^{-2}
Urine	$> 10^{-1}$
Alfalfa leaf	7.9×10^{-3}
Alfalfa and hay	9.9×10^{-3}
Grain mixture	2.3×10^{-2}
Grass	1.4×10^{-2}

The accuracy is ± 15% of the amount present.

*Sample from Harden B. Jones and Margaret R. White, Medical Physics Department, University of California.

HIGH-ENERGY NUCLEAR-REACTIONS PROGRAM

John M. Alexander, Donald W. Barr, Paul A. Benioff, Albert A. Caretto, Jr. Chalon L. Carnahan, Tom Clements, Frank Crosby, M. Frances Gallagher, James K. Grover, Earl K. Hyde, Inge-Marie Ladenbauer, David R. Nethaway and Lester Winsberg

General surveys have been made here of the products resulting from the interaction of 6-Bev protons and a number of elements: beryllium to aluminum,[1] copper,[2] tantalum,[3] and uranium.[4,5] In the region from 0.5 to 3 Bev similar surveys have been made at Brookhaven with aluminum,[6] copper,[7] lead-bismuth,[8] and uranium.[4]

From these results we are beginning to obtain an over-all picture of high-energy nuclear reactions in complex nuclei. The highest cross sections of the heavier products are in the mass region just below that of the target. The sum of the isobaric cross sections drops rapidly for smaller atomic masses until, in the broad region about half the mass of the target nucleus, it reaches a minimum value of around 10 mb. Uranium, however, is an exception; it has a broad hump in this mass region with a maximum of 30 to 40 mb. In the very lightest mass region the sum of the isobaric cross sections rises rapidly, until, in the case of protons and neutrons, the production cross section becomes greater than the geometrical cross section of the target nucleus.

The work by the Chemistry Division in the high-energy field is now turning towards a more definitive investigation of some aspects of these high-energy reactions. The new program can be roughly divided into two parts. One part is the study of the simplest reactions, such as p, pn; p, 2p; p, n; p, p'; p, $p\pi^+$; p, pxn; etc. Experiments of this type that have been completed here, or are now in progress, are the interaction of protons having energies in the Bev range with manganese,[9] indium,[10] and iodine.[11] Studies

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[1] Paul A. Benioff (to be published as a UCRL report).

[2] Donald W. Barr, UCRL-3793 (1957).

[3] James R. Grover (to be published as a UCRL report).

[4] Rex H. Shudde, Fission of Uranium with 5.7-Bev Protons (thesis), UCRL-3419, June 1956.

[5] Chalon L. Carnahan (to be published as a UCRL report).

[6] Friedlander, Hudis, and Wolfgang, Phys. Rev. 99, 263 (1955).

[7] Friedlander, Miller, Wolfgang, Hudis, and Baker, Phys. Rev. 94, 727 (1954).

[8] Wolfgang, et al., Phys. Rev. 103, 394 (1956).

[9] M. Frances Gallagher and Lester Winsberg (to be published as a UCRL report).

[10] David R. Nethaway, Excitation Functions for Reactions of Bev Protons on Indium, UCRL-3628, Jan. 1957.

[11] Ladenbauer, Alexander, and Winsberg (UCRL) (in progress).

of p, pn reactions in the energy range of 0.5 to 3 Bev have been made at Brookhaven.[12,13] The interpretation of these results in terms of the initial nucleon-nucleon collisions that occur when a high-energy proton enters a nucleus is being made by a group of collaborators at Los Alamos, Brookhaven, and the Universities of Chicago and Columbia[14] and here in Berkeley.[15] These calculations are being made with the assistance of high-speed computers by means of the Monte Carlo method, although some aspects of the problem are being solved analytically.[16]

The other part of the program consists of the investigation of the mechanism by which the products with a mass much less than that of the target are formed. We are especially interested in the products of high cross section in the mass region ~75 to ~225 formed in uranium bombardments. Since most of this "extra" cross section comes from the formation of neutron-excess nuclides, a mechanism involving fission is indicated. To check this we are measuring the cross section of neutron-excess and neutron-deficient iodine nuclides formed by the bombardment of uranium with protons in the energy region from 0.5 to 6 Bev.[17] A similar study is also planned for lighter fission products.

The excitation functions for the formation of terbium-149 from gold and bismuth, which have been measured here and at Brookhaven,[18,19] suggest a mechanism consisting of an initial cascade process followed by the evaporation of nucleons and other light particles from the highly excited residual nucleus. The gold-terbium-149 reaction is useful for monitoring high-energy protons to the exclusion of low-energy particles,[20] since the onset of this reaction is around 600 Mev.

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[12] S. S. Markowitz, Ph.D. Thesis, Princeton University, 1957.

[13] Albert A. Caretto, Jr. (UCRL) (unpublished data).

[14] Bivens, Metropolis, Storm, Turkevich, Miller, and Friedlander, Bull. Am. Phys. Soc., Series II, 2, No. 1, 63 (1957).

[15] Tom Clements and L. Winsberg, in Chemistry Division Quarterly Report, UCRL-3710, March 1957, p. 84.

[16] Lester Winsberg (UCRL) (in progress).

[17] Alexander, Gallagher, and Winsberg (UCRL) (in progress).

[18] R. B. Duffield and G. Friedlander (unpublished data).

[19] Lester Winsberg (to be published as a UCRL report).

[20] F. G. Crosby and L. Winsberg (unpublished data).

The excitation functions for fluorine-18 and sodium-24 from several target elements have also been measured here and at Brookhaven.[21] These excitation functions resemble those of p-p and n-p inelastic scattering, i.e., nucleon-nucleon encounters which result in meson formation. This type of observation has led to the proposal of a new mechanism, called fragmentation, which results from the release of π mesons in the first stage of the interaction.[8]

The recent return to operation of the 184-inch cyclotron will enable us to extend our work into the energy range below 750 Mev with high bombarding intensities and with an external beam both of which are not available at the Bevatron. We plan to make recoil and perhaps photographic emulsion studies in addition to the type of cross section measurements mentioned above.

[21] A. A. Caretto and J. Hudis (unpublished data).

CHEMICAL ENGINEERING (PROCESS CHEMISTRY)

Coalescence Rates in Two-Phase Agitated Systems

J. H. Vanderveen and T. Vermeulen

A new calibration has been obtained for the photoelectric probe used to measure interfacial areas. Also, the dependence of minimum drop diameter upon volume-fraction of dispersed phase has been found to involve mixing speed and dispersed-phase viscosity. Further progress has thus been made in developing new correlations for both the minimum diameter and the extent of coalescence.

Performance of Sphere-Packed Extraction Columns

G. Jacques and T. Vermeulen

The measurement of dispersion coefficients has been confirmed. For single-phase flow, longitudinal dispersivities were obtained for Raschig rings (3/4 inch, 1/4 inch), Intalox saddles (3/4 inch), and sand (0.45 mm average diameter). In two-phase flow, continuous-phase longitudinal dispersivities were measured for one additional random packing (0.25-inch plastic spheres) and three ordered arrangements of 0.75-inch stoneware spheres. Dispersed-phase diffusivity was studied in one random and one ordered arrangement of 0.75-inch spheres. Exploratory measurements were also made of radical dispersion in the continuous phase.

Correlation of these results is in progress. The data obtained will be of great practical use in connection with the column-performance theory now being developed (next item).

Theory of Longitudinal Dispersion

T. Miyauchi, Alice K. McMullen, and T. Vermeulen

An analytical solution has been derived for raffinate- and extract-concentration profiles under conditions in which the partition ratio, flow rates, mass-transfer coefficient, and dispersion coefficients for continuous and dispersed phases are all constant. The evaluation of this solution is being programmed, to be run on IBM 701 computer. Simpler mathematical solutions have been found for various limiting cases. A report has been written (UCRL-3911) covering the algebraic portion of this work.

Extraction rates for Single Drops

T. Miyauchi and T. Vermeulen

Uranyl nitrate extraction into penta-ether droplets has been measured at various aqueous uranium concentrations. Interpretation of these data is under way, to determine the rate-controlling step and the pertinent rate coefficients.

Zone Melting

W. R. Wilcox and C. R. Wilke

Equipment for the zone melting of naphthalene and β-naphthol mixtures has been completed and tested. Several runs at low-speed zone travel have been made and the analysis of them is under way.

A brief survey of the pertinent literature has also been made.

Stability of Perforated-Plate Trays

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

The new apparatus for studying the dumping rates from plates with one to seven holes has been built. Pressure fluctuations under the plate have been measured during unstable operation of plates having one, three, and seven holes of 0.25 inch diameter or 1 inch triangular spacings. The data show that the dumping rate is definitely related to the pressure fluctuations.

Gas-Liquid Partition Chromatography (GLPC)

R. H. Houston and C. R. Wilke

The initial phase of this work has been completed and writing of the report is in progress. Additional studies of eddy diffusion in column packings are under way.

Multicomponent Distillation Studies Using an IBM 701 Two-Frame Digital Computer

J. H. Duffin, D. N. Hanson, and C. R. Wilke

The steady-state performance of industrial distillation columns was simulated by computer, and trouble with convergence was experienced in the Thiele-Geddes method. To get around these difficulties an unsteady-state approach to the problem was worked up and programmed for the computer. It is currently being put on IBM cards preparatory to trial runs on the IBM 701 computer.

Diffusion in Nonideal Binary Solutions

Walter Wilson and C. R. Wilke

Measurements of diffusion constants for water-benzene and water-methyl ethyl ketone are in progress. Existing theoretical interpretations are being applied to the data obtained here and to other data available in the literature.

Correlation of Limiting Currents at Horizontal Electrodes under Free-Convection Conditions

E. J. Fenech and C. W. Tobias

Experiments are under way to investigate the change of convective currents with changes in geometry--i.e., length of electrode and free height above the electrode--and with changes in solution properties. The best available method of observation has been found to be the suspension of fine particles of a reflective material. At present mica is being used. Attempts at an improved method using polarized light and mica particles have been made but so far no improvement has been observed.

The length of time for steady state to be achieved in a 0.25 M $CuSO_4$ solution has been observed to be of the order of 1 minute, and in a 0.01 M $CuSO_4$ solution more than 15 minutes, thus verifying a basic assumption in Fenech's M.S. thesis, Correlation of Limiting Currents at Horizontal Electrodes under Free-Convection Conditions, UCRL-3586.

Electrochemical Studies in Nonaqueous Solvents

W. S. Harris and C. W. Tobias

Further quantitative measurements have been made of the solubility and conductivity of inorganic salts in additional solvents. The solvents now under investigation include ethylene carbonate, propylene carbonate, γ butyrolactone, and cyclopentanone.

Electrical Conductivity of Two-Phase Systems

R. E. Meredith and C. W. Tobias

Equipment is being designed and constructed for the purpose of studying the electrical conductivity of immiscible liquids as a function of the volume fraction of the dispersed phase. Experiments to date have consisted of a preliminary study of the possible liquid-liquid-electrolyte combinations that are suitable for covering a wide range of conductivity ratios.

Correlation of Heats and Free Energies of Formation of Simple Inorganic Compounds

H. Anderson and L. Bromley

Preliminary correlations based on a modification of the concepts of Pauling's electronegativity appear promising. These ideas are being tested by use of the best data available. The calculations are being made on the IBM 650 and IBM 701 computers.

Boiling Inside a Tube with High-Velocity Forced Convection

R. Wright and L. Bromley

Equipment has been designed and parts ordered for this project.

Reports Issued

A report by LeRoy Bromley, "Prediction of Performance Characteristics of the Hickman-Badger Centrifugal Boiler Compression Still," has been issued as UCRL-3874, July 1957.

GENERAL CHEMISTRY

Leo Brewer, Robert E. Connick, and Kenneth S. Pitzer in charge

METALS AND HIGH-TEMPERATURE THERMODYNAMICS

Spectroscopic Studies of C_2 and C_3

John Engelke

An arc-discharge tube has been constructed to be used in a search for electronic band systems of C_2 in the infrared. A modulated absorption system has been constructed for the King furnace to be used in a study of the ultraviolet C_3 continuum.

Thermodynamic Calculations

Elizabeth Brackett

The evaluation of the vaporization data of the dihalides of the elements has been completed and is being written up for a final report.

New Light Sources

Earl Worden

An arc for production of strong CN emission has been constructed and intensity measurements will be made to check its brightness.

Absolute Lifetime Apparatus

Richard Brewer and Fred Stafford

The electronic detection system and molecular-beam apparatus have been brought to the state at which measurements can be made on test substances such as I_2 and gaseous iron.

Temperature Scale at High Temperatures

J. Engelke, E. Worden, R. Brewer, and F. Stafford

Checks of our temperature scale in the 2000-to-3000°K region have been made with the Argonne Laboratory, the Livermore Laboratory, and U. S. National Bureau of Standards, as well as with the Physikalische Technische Reichsanstalt through secondary standards furnished by Lick Observatory. Serious discrepancies have been uncovered. These discrepancies were reported to the Union of Pure and Applied Chemistry in Paris and an international effort is planned to eliminate these discrepancies.

BASIC CHEMISTRY

Ruthenium Chemistry

Howard Cady and Dwight Fine

Part of this work has been completed and reported.[1] Chloride complexes of trivalent ruthenium are being investigated, in particular the neutral (uncharged) species. Evidence for several such neutral species has been obtained in hydrochloric acid solution. Relationships between these species are being studied, and efforts are being made to obtain these species in quantities suitable for molecular-weight determinations.

The Entropies of Dilution of Strong Electrolytes in Water

Robert Wood

A report on a correlation of the entropies of dilution of strong electrolytes is being written.

Kinetics of Rapid Reactions

Claude Coppel

The kinetics of the ferric chloride complexing reaction are being studied. The mechanism appears to be similar to that of the ferric thiocyanate system studied earlier, i.e., a path with first-order ferric-and chloride-ion dependence and a base-catalyzed path with a first-order hydroxide dependence in addition to first-order ferric- and chloride-ion dependencies. However, the acid-dependence data are not clearly resolved yet. Temperature-dependence studies have been carried out at one acidity so far.

Chemistry of Iodine in the +1 Oxidation State

Y. T. Chia

The +1 iodine species was prepared by adding hypochlorite to a basic iodide solution or by adding triiodide to soduim hydroxide. The species thus formed was unstable with respect to disproportionation, and the absorption spectrum was investigated as a function of time. When the effective molar-extinction coefficient at zero time at both 360 mμ and 400 mμ was plotted against the ratio of the initial concentrations of iodide to hypochlorite, it was found that the former varied linearly with respect to the latter from zero iodide concentration up to the point where the initial concentrations of iodide and hypochlorite were equal. However, in the region where the ratio of initial concentrations of iodide to hypochlorite changed from 1 to 5, the effective molar-extinction coefficient remained constant within experimental error. As a consequence, it was concluded that +1 iodine was the main species formed in both methods of preparation mentioned above.

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[1] Howard H. Cady, Aqueous Chemistry of Ruthenium in the +2, +3, and +4 Oxidation States: Species, Spectra, and Potentials (thesis), UCRL-3757, June, 1957.

In addition to the spectrophotometric study of the rate of disproportionation of +1 iodine, arsenite titration was used simultaneously. The rates obtained by these two methods agreed with each other within experimental error. The investigation of the rate law is in progress.

Species in Refluxed Chromic Nitrate Solutions

James Finholt

Many, if not all, multivalent metal salts form a number of different hydrolysis products in water. These products usually react rapidly with one another and water to give an equilibrium mixture. Thus one cannot isolate individual species for investigation, which makes it hard to study these solutions. Fortunately there are some metals, such as chromium, whose compounds react very slowly.

Using ion-exchange columns, we have isolated millimole quantities of three species from a refluxed chromic nitrate solution. Visible and ultraviolet spectra have been measured. From ion-exchange-column and batch-equilibration experiments now in progress we hope to learn the charge on each species, and the number of chromium atoms in each species.

Low-Temperature Heat Capacity of Ag_2O

Roger Gerkin

A cryostat with ancillary equipment for measurement of heat capacities from hydrogen temperature to room temperatue has been established. The apparatus employs a gold calorimeter equipped with an external resistance thermometer-heater for introduction of heat and determination of temperature intervals, and a strain-free platinum thermometer mounted in an axial re-entrant well for determination of absolute temperatures.

The triple point of water has been selected as the fixed point at which the stability of the strain-free thermometer will be checked; equipment for these measurements has been completed, and the triple-point resistance of the thermometer determined.

Several samples of Ag_2O of $\sim 10^2$ g consisting of "large" crystals have been prepared in accordance with the technique developed previously in this research.

NMR Study of Hydrogen Bonding

Jefferson C. Davis, Jr.

Chemical shifts in the nuclear magnetic resonance spectra of formic acid and benzoic acid solutions in benzene have been studied over a range of concentrations and temperatures. Because of the strength of the hydrogen bonds involved in the formation of the dimeric species of these acids it has not been possible to study solutions dilute enough to permit direct calculation of the equilibrium constants for the monomer-dimer systems. However, by use of the reported equilibrium constants it has been possible to calculate chemical shifts characteristic of the monomeric and dimeric species consistent with the observed shifts at various concentrations. The

magnitudes of these shifts indicate that the hydrogen bonds in the benzoic acid dimer are slightly stronger than those in formic acid, a view consistent with the reported heats of dissociation. More interesting are the shifts calculated for the monomers. The calculated monomer shifts are at a considerably higher field than shifts normally observed in proton spectra. The magnitudes of the shifts suggest that the benzoic acid momomer proton is more shielded than that in formic acid, which follows the observation that the latter is a stronger acid. These studies are being continued with the aim of confirming the monomeric shifts.

Dispersion Forces Between Atoms and Molecules

Wilm E. Donath

The work conducted here has been concerned mainly with an elucidation of the various theoretical formulae for the dispersion force between atoms and molecules. Of particular interest is the London formula,

$$E_L = -3/4\, R^{-6}\, \alpha^2\, I$$

(where α is the atomic polarizability, R the separation of the atoms, I the ionization potential, and E_L the dispersion energy). In general, this formula disagrees with experimental data by a factor of about 2, except for He, H_2, and perhaps Hg, where it is in rough agreement. Theoretical considerations suggest that the factor I should include a term resulting from the interactions of the electrons with one another in the polarized states, i.e., the field exerted from one polarized orbital will tend to depolarize all the others. This effect would appear in I as a larger promotional energy, and would make I larger as more electrons are engaged. In most cases six or eight electrons are polarized per atom; exceptions are He, H_2, and Hg, where the chief work is done by only two electrons. This seems to provide a qualitative corroboration of the theory.

Furthermore, the theoretical basis and approximations inherent in the Kirkwood-Mueller and Kirkwood-Slater formulae are being considered.

Determination of the Molecular Structure of Aluminum Hydride

Robert Nickerson

During the quarter just ended, the investigation of the procedure for the preparation of ether-free aluminum hydride was completed. The results imply that ether-free aluminum hydride cannot be prepared by the standard procedure of reacting lithium aluminum hydride with aluminum chloride in ethyl ether. In every case, regardless of the procedure used, ether was present in the preparation of aluminum hydride, always very close to the stoichiometry $AlH_3 \cdot 0.25\ Et_2O$. A possible explanation is that the $AlCl_3$ forms a strong Al-O bond to an ether molecule forming the complex $AlCl_3 \cdot Et_2O$, and that when this complex reacts with lithium aluminum hydride, the ether is retained. Hence the reaction

$$LiAlH_4 + AlCl_3 \cdot Et_2O \xrightarrow{\text{ether}} LiCl\downarrow + 4\ AlH_3 \cdot 0.25\ Et_2O.$$

As a test for this hypothesis, a sample of aluminum hydride was synthesized by utilizing the reaction

$$4LiAlH_4 + SiCl_4 \xrightarrow{\text{ether}} LiCl\downarrow + SiH_4\uparrow + 4AlH_3.$$

The aluminum hydride produced in this manner appeared to be ether-free, but the compound was unstable and decomposed when placed under vacuum. A sample of the gas evolved contained less than 1% ether.

Heat Capacities of Metals at Low Temperatures

Norman Phillips

The measurements on Cu and Al have been extended and now cover the range between 0.25° and 4.2°K. At temperatures below about 0.65° the heat capacity of superconducting Al can be represented by $C = 7.6\ \gamma\ T_c \exp(-1.32\ T_c/T)$, where γ is the coefficient of the linear term in the normal state heat capacity and T_c is the transition temperature, which was found to be 1.171°K.

Some preliminary investigations on heat transfer in a fine powder suggest that the apparatus may be useful for an investigation of particle-size effects on the heat capacity at He temperatures.

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