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BIO-ORGANIC CHEMISTRY
QUARTERLY REPORT
September, October, November 1959

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Contents

Centrifugally Accelerated Paper Chromatography of Chloroplast Pigments
Joan Mary Anderson 3

D-Threose 2, 4-Diphosphate Inhibition of 3-Phosphoglyceric Acid Photoreduction by a Sonically Ruptured Chloroplast System
Roderic B. Park, Ning G. Pong, Karel P. Louwrier, and M. Calvin . . 12

The Biosynthesis of Opium Alkaloids
Frank R. Stermitz and Henry Rapoport . 22

Investigation of Organic Matter in Meteorite Murray
Susan K. Vaughn and Melvin Calvin . . 24

Strain Differences in Acetyl Choline Concentration in Rat Brain
Edward L. Bennett, Marie Hebert, Ann Ohlander, and Patricia Smith . . 35

Unpaired Electrons in Systems Bound by Charge-Transfer Forces
John W. Eastman and M. Calvin . . 40

Proton Magnetic Resonance in Simple Polypeptides
Tatsumi Ohki 47

Preliminary Studies on the Occurrence of Manganese Phaeophytin in Plants
Edward Markham 55

Hydrogen Bonding Studies by NMR Methods
Irwin D. Kuntz, Jr. 58

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BIO-ORGANIC CHEMISTRY QUARTERLY REPORT

September, October, November 1959

M. Calvin, Director

Edited by Edward L. Bennett

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

December 21, 1959

CENTRIFUGALLY ACCELERATED PAPER CHROMATOGRAPHY OF CHLOROPLAST PIGMENTS

Joan Mary Anderson

The techniques of centrifugally accelerated paper chromatography were developed by G. Caronna in 1955[1] and H. J. McDonald in 1957.[2] The combination of two vectors, chromatography and centrifugal acceleration, makes it possible to separate compounds in very short times, for example in 15 minutes instead of 8 hours. The chlorophylls and carotenoids are very labile compounds which undergo marked decomposition in the lengthy paper chromatography necessary for adequate resolution. It was hoped that this difficulty might be overcome by the use of rapid centrifugally accelerated paper chromatography.

The literature reports separations of water-soluble compounds, such as dyes and amino acids,[3] but as yet there is no mention of separation of any other compounds. Therefore, a study was made of the fundamental factors involved in obtaining good separations and reproducible chromatograms of the algal pigments with nonpolar solvents.

Experimental

The centrifugally accelerated chromatograms were made on the chromatofuge which was assembled and operated in the prescribed manner.[4] All the experiments reported were carried out at 25°C. Whatman No. 1 and 3MM circles were used, as well as circles cut from the very thick Whatman No. 17 MM sheets. Since most of the pigments were required for spectroscopic and radioactive measurements or for further chromatography,

---

[1] G. Caronna, Chim. e Ind. (Milan) 37, 113 (1955); Chem. Abstr. 49, 7299d (1955).

[2] McDonald, Bermes, and Shepherd, Chromatog. Methods 2, 1 (1957).

[3] McDonald, McKendell, and Bermes, J. Chromatog. 1, 259 (1958).

[4] Labline No. 5060 Chromatofuge, Labline Inc., Chicago, Illinois.

it was desirable to use the thicker Whatman Nos. 3 MM or 17 MM papers. The pigment extract was placed on a circular origin which must be at least 8 cm in diameter. It was most satisfactory to apply the pigments in ethereal solution, as the ether evaporated quickly; however, other solvents were also feasible. With practice it was possible to make very thin uniform circles of pigment on the origin by holding the paper vertically with one hand and applying the extract with the other; papers were spotted in the cold room in the presence of as little light as possible.

The total algal extract that could be placed on such a circular origin on a Whatman No. 3 MM sheet was equivalent to 0.3 to 0.4 mg pigment, i.e., the extract obtained from 0.05 ml wet packed algal cells. About three times as much material could be placed on the circular origin of Whatman No. 17 MM paper. Since the paper was thick it was necessary to spot the material on both sides, otherwise the pigment zones on the underside of the paper after development would have lower R_F values.

It was also desirable to use a liner for the chromatofuge head. A sheet of Whatman No. 3 MM paper was placed in close contact with the bottom of the head, by cutting holes in the sheet so that the stainless steel points protruded through the liner paper. Before placing the paper to be developed in the chromatofuge, some of the solvent was placed on this liner and the machine rotated for 5 minutes in order to saturate the chamber head with the solvent fumes. In subsequent runs, about 10 to 20 ml of solvent was poured onto the liner, the paper placed in position, and developed. It was not necessary, in the subsequent "run," to rotate the machine for the 5-min period before the development of the paper.

With petroleum-ether-isopropanol solvents, it was necessary to mix the solvent several days before it was required for use. Fresh solvent mixtures used under the same running conditions gave anomalous results (the solvent did not travel nearly as far and the separations obtained were very poor).

Results and Discussion

First of all it should be pointed out that the chromatograms obtained, despite the velocity of the rotation, are not circular in shape but elliptical, as was reported by McDonald et al. for aqueous solvents.[3] This is not a disadvantage, however, since measurement of R_F values from different parts of the ellipse gave values that were within the experimental error (± 0.02 R_F units).

It was found that the resolution of the carotenoids and chlorophylls was the same as that obtained in conventional ascending or descending chromatography; the latter methods have been reviewed by Sestak.[5] That is, any solvent that gave satisfactory resolution in the conventional methods

[5] Z. Sestak, J. Chromatog, 1, 293 (1958).

could be used for the centrifugally accelerated chromatograms, although it was necessary to determine suitable running conditions for the chromatofuge.

Most of the factors reported by McDonald et al.[3] for development of the chromatograms with aqueous solvent were found to be effective in these experiments with nonpolar solvents. In order to obtain reproducible R_F's it is necessary to have a flow rate of solvent such that the perimeter of the wetted area does not increase after the rotor and solvent flow are stopped. It is also necessary to avoid such a high flow rate that flooding of the paper occurs during development, as this causes distortion of the zones.

The placement of the circular origin is important; it must not be less than 7.5 cm in diameter or else some of the pigments migrate inwards. It was found that an increase of 1.0 cm for the diameter of the circular origin resulted in an increase of about 0.04 for intermediate R_F values. It was not advantageous to increase the diameter beyond 12 cm, since under those conditions only spots with R_F values less than 0.3 would be separated.

In general, under the same conditions of nitrogen pressure and velocity of rotation, and with the same delivery jet, different solvents travel different distances, as might be expected. For example, at 500 rpm, 7.5 psi of nitrogen, and a flow rate of 2.0 ml/min, toluene traveled 15 cm; petroleum ether (bp 75°), 13 cm; petroleum ether (bp 30-60°), 6 cm; and petroleum ether–isopropanol (100:2.5) 14 cm in 10 minutes.

Also, with different grades of paper the distance traveled by the solvent varies. With a flow rate of 2.5 ml/min of toluene at 500 rpm results were as follows.

Paper	Time of development (min)	Distance from origin to solvent front (cm)
Whatman No. 1	10	16
	12.5	17.5
	15	19
Whatman No. 3 MM	10	14
	12.5	15
Whatman No. 17 MM	10	6
	20	14
	25	16

It can be seen that increasing the time results in a nonlinear extension of the solvent front. Obviously, in trying out a new grade of paper or a different solvent, it is necessary to make some trial runs with no pigments on the paper to find a satisfactory time.

Yet another variable needs to be considered: the use of the liner paper. This is essential with most solvents, in particular with petroleum ether solvents. Even with the heavier Whatman Nos. 3 MM or 17 MM circles, it is necessary to have a liner.

Table I illustrates the R_F values obtained, by using the same solvent and conditions, at different velocities. In general, it is seen that above 450 rpm, the R_F values decrease as the velocity of rotation increases, although for a change of 50 rpm the decrease in the R_F is within the experimental error. Changes obtained over the complete range of velocities available (i.e., 350 to 950 rpm) are significant, however. It will be noticed that between 350 and 450 rpm the R_F values remain the same or increase with increasing velocity, while the general pattern in the range 450 to 950 rpm is one of decreasing R_F values. The speed at which the R_F values are a maximum is termed the "optimum speed"; the "optimum speed" in Table I is, therefore, about 450 rpm.

For several other solvents a similar set of runs was made at varying velocities and the same type of pattern was found as shown in Table I. Instead of listing the whole gamut of R_F values obtained, Table II shows the R_F values at the "optimum speed." It is seen that this optimum speed is not constant for different solvents, but it seems to lie nearer the lower velocities of rotation.

Comparison of the R_F values listed in Tables I and II to those obtained in the conventional ascending or descending chromatography show the similarity obtained with the same solvents. In all cases carotene runs right at the solvent front. Solvents with a higher proportion of toluene are useful for the separation of the carotenoids; on the other hand, the chlorphyll a and b travel further in petroleum ether–isopropanol solvents. Figures 1 and 2 show the widths of the pigment zones obtained; it is seen that the bands are very narrow and well-defined. They are less than one-half as wide as those which result from descending or ascending chromatograms that have been run the same distance as the chromatofuge chromatograms.

In the hopes of obtaining better separation of chlorophyll a and b, reverse-phase chromatography was tried. The methanol used as the solvent for the development of the paraffin-oil-impregnated paper showed a great tendency to flood the papers. A flow rate of 1.25 ml/min with 4 psi of nitrogen pressure was necessary, which meant at least 15-min development time. The results obtained were disappointing; the bands were diffused and streaking into one another. All the pigments other than the carotene, which stayed right at the origin, had R_F values greater than 0.6, which meant that they were all crowded together despite the variety of conditions tried.

Table I

Chromatofuge R_F values of algal pigments

Conditions: Whatman No. 3 MM paper (unwashed). Solvent, petroleum ether–isopropanol (100:2.5); flow rate of 2.5 ml/min; 7.5 psi nitrogen pressure; temp. 25°C; liner saturated with 20 ml of solvent; time of development 10 min; circular origin 10 cm. The R_F values reported are the average of 3 separate runs at each velocity quoted.

Compound	Speed (rpm)									
	350	400	450	500	550	600	650	750	850	950
carotenes	0.91	0.92	0.94	0.94	0.96	0.95	0.97	0.96	0.97	0.98
lutein	0.46	0.47	0.56	0.47	0.44	0.42	0.36	0.35	0.32	0.30
chlorophyll a	0.44	0.45	0.47	0.38	0.37	0.30	0.26	0.27	0.23	0.22
chlorophyll b	0.27	0.26	0.26	0.23	0.23	0.16	0.14	0.14	0.11	0.08
neoxanthin	0.06	0.08	0.10	0.13	0.11	0.11	0.08	0.05	0.05	0.04

Note: As in ascending or descending chromatograms, with petroleum-ether–isopropanol (100:2.5), violaxanthin is masked in the bottom of the chlorophyll a spot.

Table II

Chromatofuge R_F values of algal pigments at optimum speed[a]

Conditions: Whatman No. 3 MM paper (unwashed); flow rate 2.5 ml/min; nitrogen pressure 7.5 psi; liner saturated with 20 ml of solvent (unnecessary for toluene); time of development 10 min; circular origin, 10 cm. R_F values reported are the averages of three separate runs at the velocities quoted.

Compound	Solvent and optimum speed							
	Toluene 600 rpm		Toluene - Pet. ether, 4:1 500 rpm		Toluene - Pet. ether, 1:1 750 rpm		Toluene - Pet. ether, 1:3 500 rpm	
	Total extract[5]	Carotenoid extract[5]	Total extract	Carotenoid extract	Total extract	Carotenoid extract	Total extract	Carotenoid extract
carotene	0.96	0.97	0.98	0.97	0.98	0.94	0.98	0.95
lutein	0.89	0.90	0.90	0.90	0.83	0.85	0.75	0.73
violaxanthin	0.82	0.80	0.78	0.74	0.70	0.50	0.48	0.35
chlorophyll a	0.34		0.37		0.33		0.30	
chlorophyll b	0.19		0.27		0.22		0.19	
neoxanthin	0.04	0.06	0.07	0.07	0.10	0.07	0.12	0.17

[a] "Optimum speed" is defined as that velocity of rotation of the chromatofuge for which the R_F values of the pigments are a maximum.

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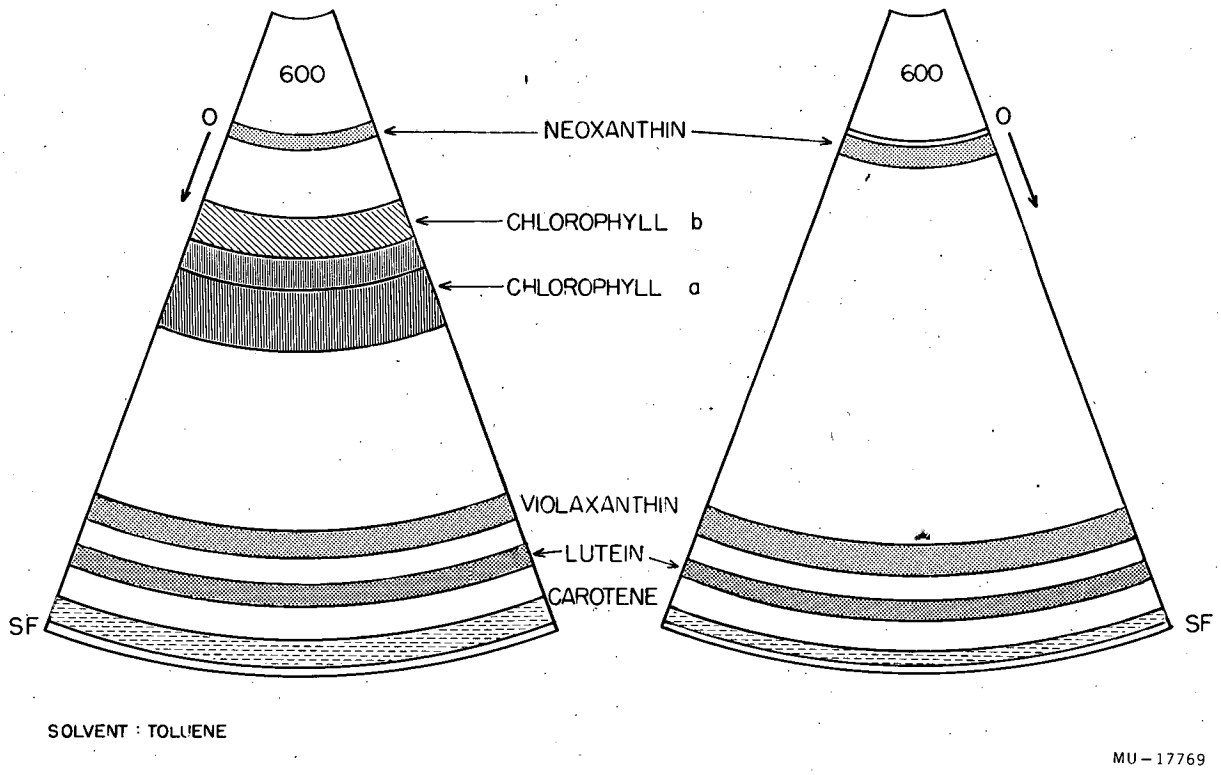

Fig. 1. Sectors of centrifugally accelerated paper chromatograms of the total pigment and carotenoid extracts from Chlorella using toluene as the solvent at 600 rpm. Time of development, 12.5 min; flow rate, 2.5 ml/min; origin to solvent front, 17 cm; Whatman No. 3 MM paper.

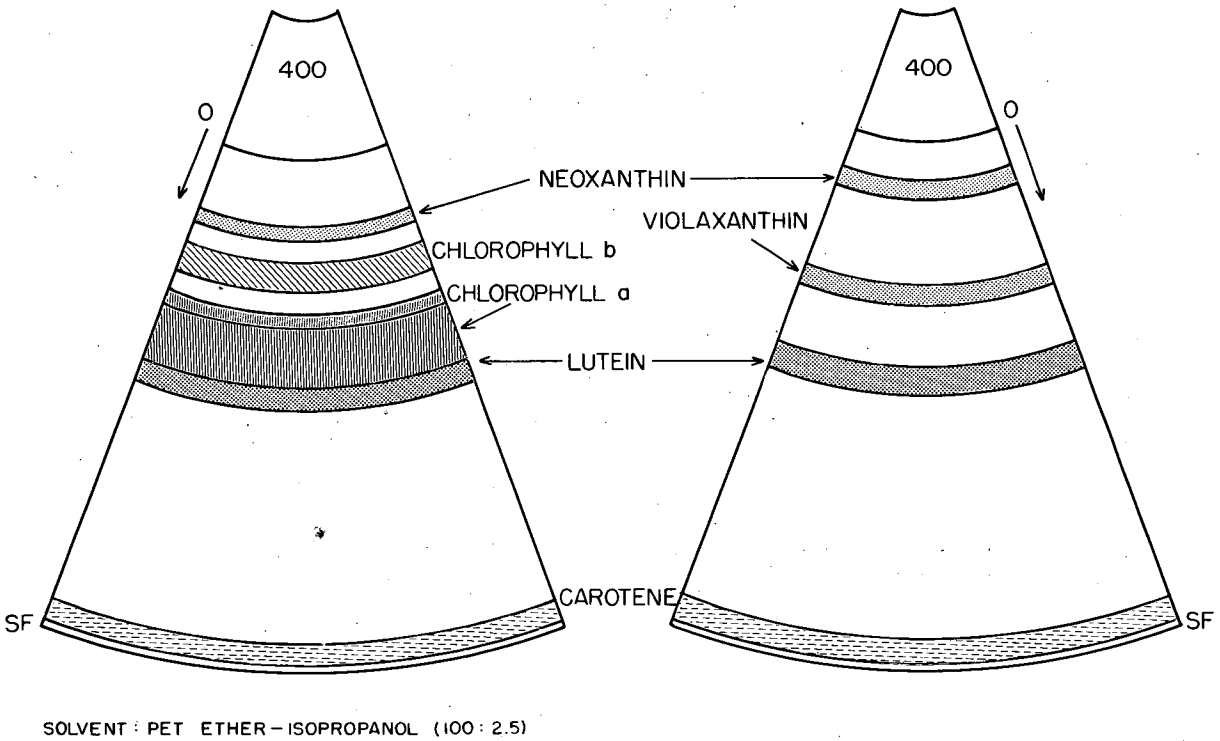

Fig. 2. Sectors of centrifugally accelerated paper chromatograms of the total pigment and carotenoid extracts from Chlorella, using petroleum ether-isopropanol (100:2.5) as the solvent at 400 rpm. Time of development, 10 min; flow rate, 2.5 ml/min; origin to solvent front, 17 cm; Whatman No. 3 MM paper.

The quantitative recovery of pigments from a typical chromatogram was checked. Pigments (0.21 mg) were placed on the origin of a Whatman No. 3 MM paper which was developed for 10 min with a flow rate of 2.3 ml/min at 500 rpm, using toluene. The spots were eluted immediately with ether and the concentrations measured spectrophotometrically. About 88% of the pigments placed on the origin were recovered, which is much better than ascending chromatography, in which the average recovery was about 70%.[6] Since the chromatograms take 10 to 15 minutes instead of 2 to 3 hours to be developed, there is less time for decomposition of the pigments. The pigment zones are easily eluted with ether and less time is needed to obtain colorless paper, hence there is a smaller loss in this operation than on the ascending or descending chromatograms. It would probably be advantageous to operate the chromatofuge at a lower temperature than 25°; it was not possible to do this with our machine.

Summary

Centrifugally accelerated paper chromatography for the separation of chloroplast pigments gives results similar to those of conventional paper chromatographic methods. This technique appears to be very promising for the separation and recovery of the labile chlorophylls and carotenoids because of the narrowness and compactness of the pigment zones and the rapid development time. The pigments were easily eluted from the paper and recovery was greater than from the usual paper chromatograms.

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[6] Joan Mary Anderson, Research in Photosynthesis (Ph. D. Thesis), UCRL-8870, Sept. 1959.

D-THREOSE 2,4-DIPHOSPHATE INHIBITION OF 3-PHOSPHOGLYCERIC ACID PHOTOREDUCTION BY A SONICALLY RUPTURED SPINACH CHLOROPLAST SYSTEM

Roderic B. Park, Ning G. Pon, Karel P. Louwrier, and M. Calvin

The first stable intermediate formed by $C^{14}O_2$ fixation in illuminated photosynthetic systems is 3-phosphoglyceric acid (PGA).[1,2] Recently, an alternative path has been suggested for photosynthetic CO_2 fixation which does not involve PGA.[3] In this suggested path, carboxylation of RuDP yields a six-carbon branched-chain acid which subsequently rearranges without cleavage to PGA, to form a six-carbon chain directly. If it were possible to block the reduction of PGA to triose in the photosynthetic carbon cycle, then it should be possible to observe whether CO_2 still flows into the cycle via some pathway other than that involving PGA.

Recently threose 2,4-diphosphate was shown to inhibit specifically muscle and yeast triose phosphate dehydrogenase having DPN as a cofactor.[4,5] This inhibitor has a K_I of 2×10^{-7} for the oxidation reaction. The reduction reaction is less sensitive to the inhibitor, a concentration of about 2×10^{-5} M yielding 50% inhibition. The inhibition of the enzyme is apparently due to the similarity of the threose diphosphate and 1,3-diphosphoglyceric acid molecules. It was of interest to determine whether threose diphosphate would also inhibit triose phosphate dehydrogenase in a TPN-requiring photosynthetic system. Such an inhibition would then make it possible for PGA to accumulate when $C^{14}O_2$ was photosynthetically fixed in the presence of threose diphosphate. Since the photosynthetic carbon cycle involves a number of enzymatic steps other than triose phosphate dehydrogenase, it was of interest to find whether some of these other enzymes were also inhibited by threose diphosphate. The experiments reported in this paper were done with a spinach chloroplast system, since phosphorylated intermediates such as PGA and threose diphosphate do not readily penetrate intact cells. Whole chloroplasts were also found to be somewhat impermeable to the phosphorylated substrates and cofactors used in these experiments,

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[1] The following abbreviations are used in this paper: ADP, adenosine diphosphate; GSH, glutathione; HOAc, acetic acid; PEP, phosphoenolpyruvate; PGA, 3-phosphoglyceric acid; RuDP, ribulose 1,5-diphosphate; SAS, saturated ammonium sulfate; TPN, triphosphopyridine nucleotide; TPP, thiamine pyrophosphate; Tris, tris (hydroxymethyl) aminomethane; DPN, diphosphopyridine nucleotide.

[2] J. A. Bassham and M. Calvin, The Path of Carbon in Photosynthesis (Prentice-Hall, Inc., Englewood Cliffs, New Jersey, 1957).

[3] O. Kandler, Z. Naturforsch. 12b, 271 (1957).

[4] Racker, Klybas, and Schramm, J. Biol. Chem. 234, 2510 (1959).

[5] A. Fluharty and C. Ballou, J. Biol. Chem. 234, 2517 (1959).

confirming the observation by Tolbert.[6] We found the sonic rupture of whole chloroplasts removed the permeability barrier and increased the rate of CO_2 fixation on a unit chlorophyll basis. Sonically ruptured chloroplasts proved more suitable for these experiments than either whole or broken chloroplasts as prepared by Arnon.[7]

Procedures

Whole chloroplasts were prepared from the leaves of Soinacea oleracea by grinding in 0.5 M sucrose buffer and were purified by fractional centrifugation according to the method described by Holm-Hansen et al.[8] The whole, washed chloroplasts were resuspended in about 20 times their own volume of 10^{-3} M potassium phosphate buffer, pH 7.4. The chloroplast suspension was then sonically ruptured at 0° C for 90 seconds in a 9-kc 100-watt Raytheon magnetostriction oscillator operated at full voltage. The sonically ruptured suspension was used directly in the experiments. Investigation of this material with the light microscope showed no intact chloroplasts, and only a few large chloroplast fragments. Chlorophyll concentrations in the sonically ruptured suspension were determined spectrophotometrically in 80% acetone according to the method of Arnon.[9]

The organic cofactors and metal cofactors were prepared separately and titrated to pH 7.4. The photosynthetic reactions were run at 24° C and 1000 foot-candles in airtight 35-ml flasks. The total reaction mixture volume was about 1.5 ml and contained about 1 mg of chlorophyll.

After 30 minutes' incubation the enzymatic reaction was stopped by addition of 4 ml of ethanol to the reaction mixture. The precipitate was then extracted with 20% ethanol, 100% ethanol, and water successively. The extracts were pooled, giving a total volume of 10 ml. A small aliquot was acidified and placed on an aluminum planchet for assay of the total radioactivity in the reaction mixture. Another aliquot was applied to the origin of a paper chromatogram.

Two-dimensional chromatography of the photosynthetic products and counting of the radioactive areas on the chromatograms were carried out according to the methods described by Bassham and Calvin.[2]

Carboxydismutase was prepared from Tetragonia expansa chloroplasts. These were prepared in 0.5 M sucrose and suspended in dilute buffer according to the method of Sogo et al.[10] Carboxydismutase was obtained by ammonium sulfate fractionation of the clear supernatant

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[6] N. E. Tolbert, The Photochemical Apparatus, its Structure and Function, Brookhaven Symposia in Biology, No. 11, 271 (1958).

[7] Whatley, Allen, Rosenberg, Capindale, and Arnon, Biochim et Biophys. Acta 20, 462 (1958).

[8] Holm-Hansen, Pon, Nishida, Moses, and Calvin, Physiol. Plantarum 12, 475 (1959).

[9] D. I. Arnon, Plant Physiol. 24, 1 (1959).

[10] Sogo, Pon, and Calvin, Proc. Natl. Acad. Sci. U.S. 43, 387 (1957).

(termed "chloroplast extract" in Ref. 10) obtained from centrifugation of the chloroplast suspension. The procedure of Mayaudon,[11] normally used for the partial purification of the crude extract of Tetragonia expansa, was modified for fractionation of the chloroplast extract, the material precipitating between 0.34 and 0.49 SAS being chosen for use in these experiments.

Carboxyl-labeled PGA was prepared by incubating RuDP with C^{14}- labeled sodium bicarbonate in the presence of carboxydismutase[12] and Mg^{++}.[13,14] The reaction mixture consisted of 12.7 μM $NaHC^{14}O_3$ (300 μC), 120 μM $MgCl_2$, 900 μM tris at pH 8.3, about 3 μM RuDP, and 1.4 mg of enzyme preparation. The final volume of the incubation mixture was 2.25 ml. Incubation was carried out at room temperature for 20 hours. The yield of radioactive PGA was about 1.5 μM, or 50% of the theoretical value. The PGA-1-C^{14} was purified by chromatography on washed No. 4 Whatman paper with a butanol–propionic acid–water system. The C^{14}-PGA was located by radioautography and was recovered by elution. The eluate was taken to dryness and redissolved in 0.5 ml of 0.040 N $NaHCO_3$.

D-threose 2, 4-diphosphate was generously provided by Dr. Ballou and Dr. Fluharty of the University of California Biochemistry Department.

Experimental Results and Discussion

Preliminary experiments were done in which PGA-1-C^{14} was incubated with the sonically ruptured chloroplast system in light and in dark with the cofactors given in Table III. Chromatograms from this experiment (Fig. 3) showed that reduction of PGA-1-C^{14} to triose phosphate in this enzyme system is a light-dependent process. In the dark PGA-1-C^{14} is not reduced. This accounts for the relatively large amounts of glyceric acid and alanine in the dark experiment. When PGA-1-C^{14} reduction was established as a light-dependent process in the system, threose diphosphate inhibition of spinach chloroplast triose phosphate dehyrogenase was investigated by incubating PGA-1-C^{14} with sonically ruptured spinach chloroplasts in light in the presence and absence of threose 2, 4-diphosphate. The reaction mixtures for this experiment are given in Table III. After two-dimensional paper chromatography of the reaction mixture extract and location of the C^{14}-containing spots by autoradiography, the activity of the PGA area was compared with the activity of mono-and diphosphate sugar areas. The results of this experiment are given in Table III. The presence of 10^{-4} M threose diphosphate increased the ratio of PGA activity to the activity of the mono- and diphosphate sugar areas, which would be expected if triose phosphate dehydrogenase were inhibited during operation of the photosynthetic cycle.

---

[11] Mayaudon, Benson, and Calvin, Biochim. et Biophys. Acta 23, 342 (1957).

[12] Quayle, Fuller, Benson, and Calvin, J. Am. Chem. Soc. 76, 3610 (1954).

[13] Weissbach, Horecker, and Hurwitz, J. Biol. Chem. 218 795 (1956).

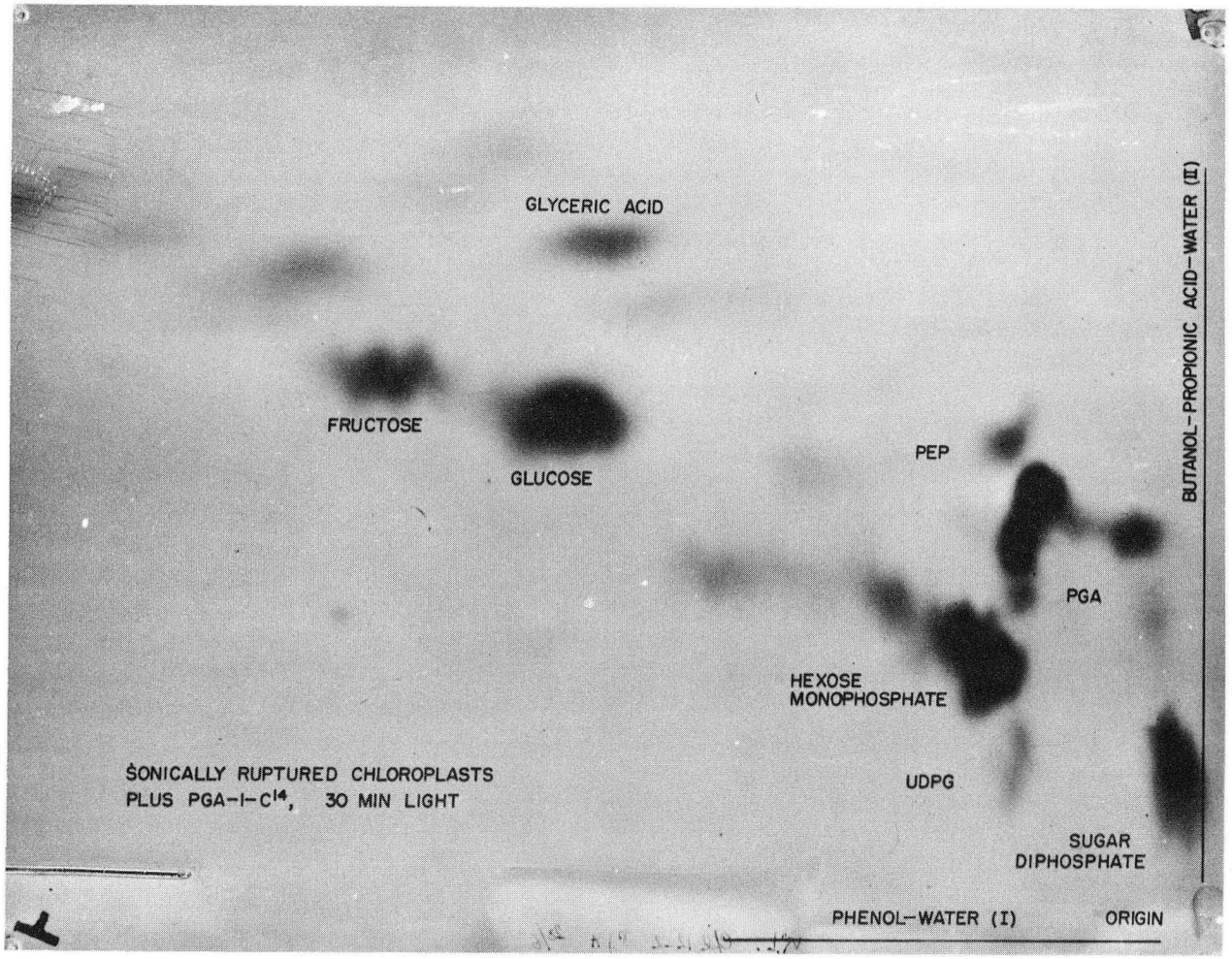
[14] Jakoby, Brummond, and Ochoa, J. Biol. Chem. 218, 811 (1956).

Table III

Distribution of C^{14} label among PGA, the monophosphate sugars, and diphosphate sugars when PGA-1-C^{14} is incubated with and without threose diphosphate in an illuminated chloroplast system.

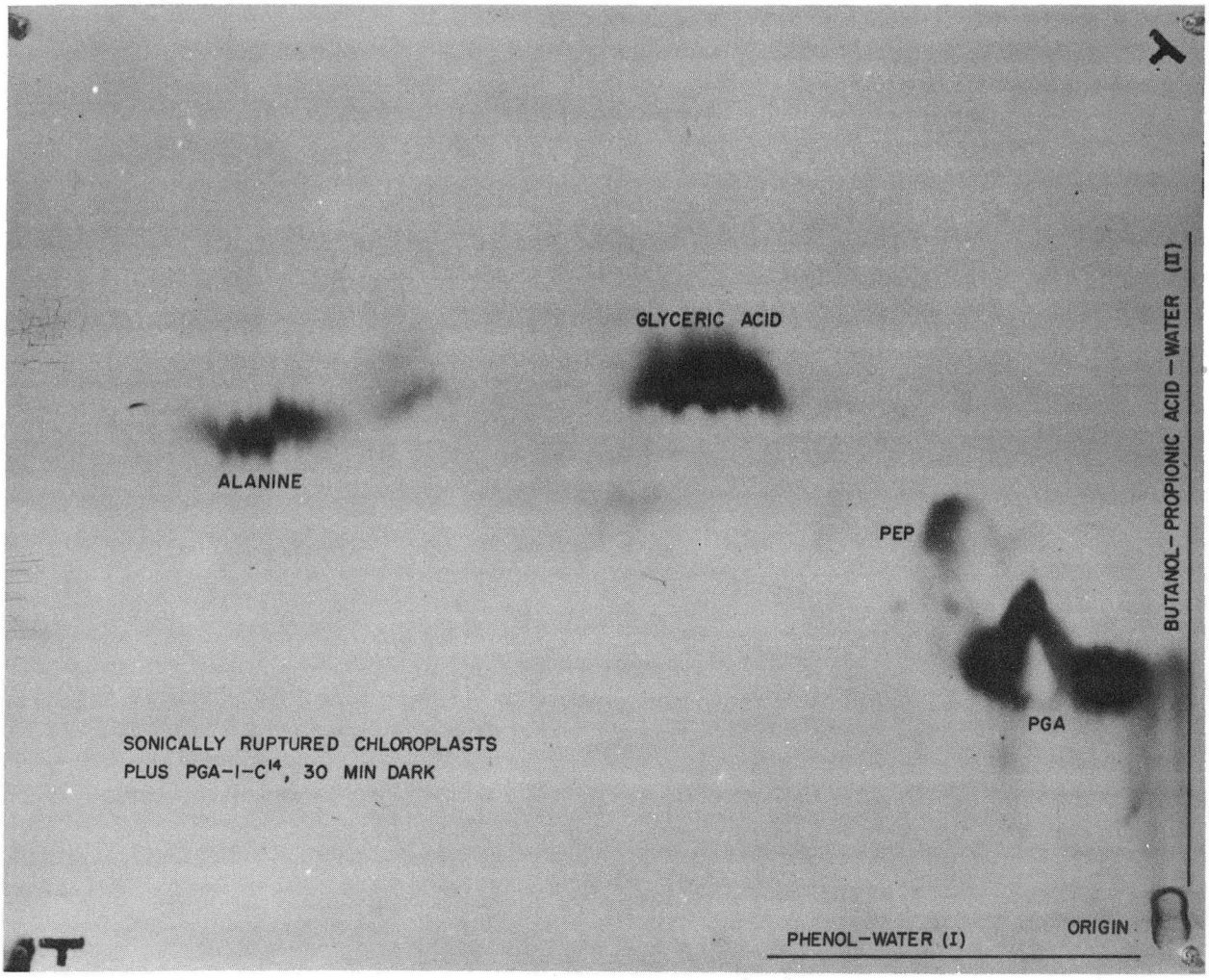
	Activity on chromatogram (cpm)	
	without threose diphosphate	with threose diphosphate
Total	27,000	23,000
PGA	5,800	12,000
Monophosphates	7,300	1,900
Diphosphates	3,100	800

Each reaction mixture contained (amount in μM) $MgCl_2$, 4.0; $MnSO_4$, 1.6; ADP, 1.0; TPN, 0.02; TPP, 1.4; ascorbate, 2.5; GSH, 1.3; $NaHC^{12}O_3$, 4.0; fragmented chloroplasts, 1 ml; PGA-1-C^{14}, 1,200,000 cpm or approximately 0.1 μM; threose 2,4-diphosphate, 0.12 (present only in one reaction mixture). Total volume 1.275 ml.



ZN-2307

Fig. 3a. Chromatograms of extracts from sonically ruptured spinach chloroplast systems incubated with PGA-1-C^{14} in light.



ZN-2306

Fig. 3b. Chromatograms of extracts from sonically ruptured spinach chloroplast systems incubated with PGA-1-C^{14} in dark.

A second experiment was done in which $C^{14}O_2$ fixation was studied in the presence and absence of threose diphosphate. The reaction mixtures were identical to those given in Table III except that 4 μM $NaHC^{14}O_3$ (100 μC) was substituted for the PGA-1-C^{14}. The results of this experiment are given in Table IV. Again there is an increase in the ratio of PGA activity to phosphorylated sugar activity in the threose-diphosphate-inhibited system.

The similarity between chromatograms of extracts from $HC^{14}O_3^-$ and PGA-1-C^{14}-incubated systems indicates that PGA is readily assimilated into the $C^{14}O_2$ pathway of the photosynthetic carbon cycle. The qualitative and quantitative similarity in the effect of threose diphosphate inhibition of the two systems further indicates that PGA is a real and major intermediate in the photosynthetic carbon cycle. Although these experiments show that $C^{14}O_2$ photosynthetically fixed by a chloroplast system flows through PGA, they do not settle the question of whether the other three carbon fragments resulting from cleavage of the RuDP carboxylation product are also PGA.[15]

An interesting feature of Table IV is that the light fixation of $C^{14}O_2$ in the presence of threose diphosphate is about one-third of the control fixation. Inhibition of any of the enzymatic steps of the photosynthetic carbon cycle to a rate-limiting level would be expected to reduce the total $C^{14}O_2$ fixation by the cycle. The location of threose diphosphate inhibition in the photosynthetic carbon cycle of spinach chloroplast fragments is shown in Table III to be at least in part at triose phosphate dehydrogenase. There is the possibility that 10^{-4} M threose diphosphate may also be affecting the rates of other photosynthetic carbon cycle enzymes. Inhibition of most of these enzymes would limit the rate of formation of the acceptor molecule for CO_2 fixation, RuDP. Inhibition of carboxydismutase, however, could also involve direct inhibition of $C^{14}O_2$ entry into the cycle rather than just limitation of ribulose diphosphate formation.

For this reason we tested the effect of threose diphosphate on carboxydismutase activity, both in the crude spinach preparation and in a purified enzyme system isolated from Tetragonia expansa. The results for these experiments are given in Fig. 4 and Table V. (Conditions: Each reaction mixture contained 4.0 μM $NaHC^{14}O_3$ (100 μC), 0.2 μM RuDP, and 1 ml of sonically ruptured chloroplasts in 10^{-3} M potassium phosphate (pH 7.4) containing 0.59 mg of chlorophyll. Mg^{++}, Mn^{++}, ADP, TPN, TPP, ascorbate, and GSH concentrations are given in Table III. The reaction mixtures minus $NaHC^{14}O_3$ were preincubated for 1 min at room temperature, after which the bicarbonate was added. The reaction mixtures were shaken in the dark for the prescribed time at 25°C in airtight 35-ml vessels. $C^{14}O_2$ fixation by the same enzyme system in light minus RuDP, expressed in 10^6 cpm/mg chlorophyll/30 min incubation, was 21 without threose diphosphate and 7.4 with 10^{-4} M threose diphosphate.) These data show that 10^{-4} M threose diphosphate has no effect on the carboxydismutase of the crude system, and inhibits the purified system by only 15%.

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

Table IV

Distribution of C^{14} label among PGA, the monophosphate sugars, and diphosphate sugars when $HC^{14}O_3^-$ is incubated with and without threose 2, 4-diphosphate in an illuminated chloroplast system.

	Activity on chromatogram (cpm per aliquot)	
	without threose diphosphate	with threose diphosphate
Total	230,000	98,000
PGA	93,000	60,000
Monophosphates	53,000	8,200
Diphosphates	23,000	7,000
Total counts fixed in the reaction mixture:		
Light	16,000,000	5,800,000
Dark	130,000	110,000

Each reaction mixture contained (amount in μM) $MgCl_2$, 4.0; $MnSO_4$, 1.6; ADP, 1.0; TPN, 0.02; TPP, 1.4; ascorbate, 2.5; GSH, 1.3; $NaHC^{14}O_3$, 4.0 (100 μC); sonically ruptured chloroplasts, 1 ml.

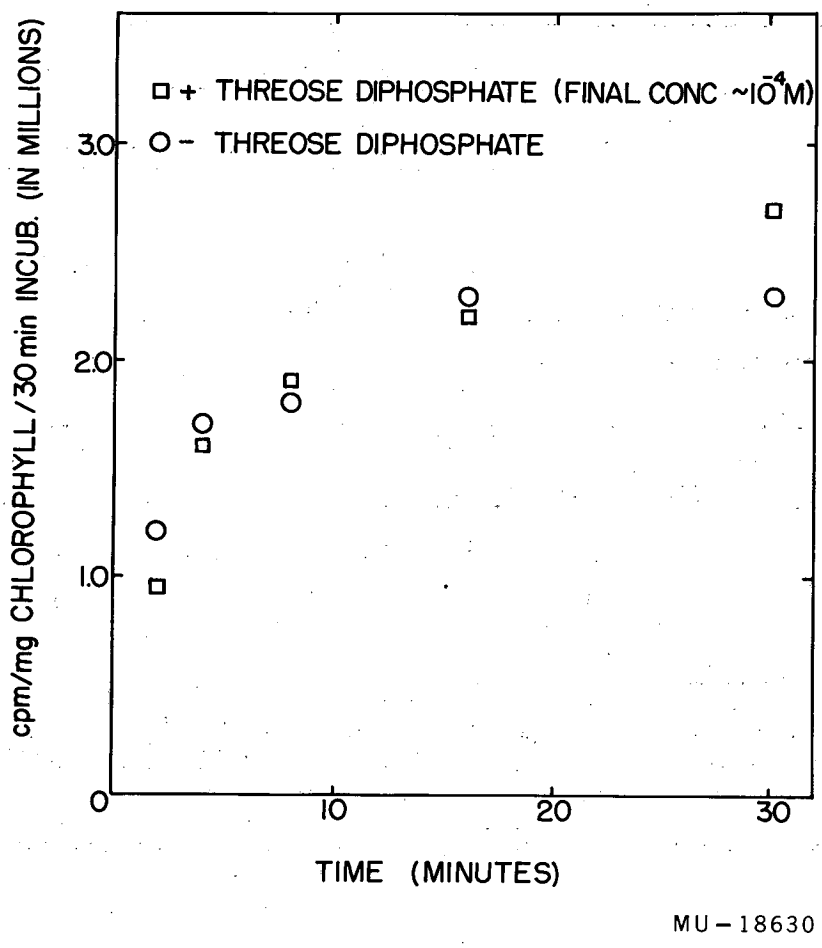


Fig. 4. The time course of $C^{14}O_2$ fixation by carboxydismutase in a sonically ruptured chloroplast system with and without threose diphosphate.

Table V

The inhibition of purified carboxydismutase by threose diphosphate

Threose diphosphate final conc. (M)	cpm per 10-µl aliquot	% inhibition
0	16,000	-
8×10^{-6}	15,000	6
8×10^{-5}	14,000	15
8×10^{-4}	7,600	53

To each reaction mixture were added, in the order listed, the following; water or threose diphosphate solution to make 0.20 ml final volume; $NaHC^{14}O_3$, 1.35 µM; $MgCl_2$, 2 µM; tris at pH 8.3, 15 µM; carboxydismutase, 23 µg; and RuDP, about 0.07 µM. The enzyme was preincubated with the inhibitor, bicarbonate, and Mg^{++} at 25°C for 2 min. At the end of this period, the incubation was started by adding RuDP. The reaction mixture was incubated for 10 min at 25°C. The reaction was stopped with 50 µl 6N HOAc. A 10-µl aliquot was plated and counted.

This result indicates that 10^{-4} M-threose-diphosphate inhibition of $C^{14}O_2$ fixation by this system is not due to inhibition of carboxydismutase, which would prevent entry of CO_2 into the cycle, but is primarily due to inhibition of triose phosphate dehydrogenase. The possibility of non-rate-limiting inhibition of some other enzymes acting between triose phosphate and ribulose diphosphate is not, however, excluded.

The data plotted in Fig. 3 also show that the carboxydismutase rate observed during the first 2 minutes of the assay is sufficient to account for the rate of $C^{14}O_2$ fixation observed in the light sample.

Summary

1. PGA-1-C^{14} is readily reduced by a sonically ruptured chloroplast system in light. This reduction is inhibited by threose 2, 4-diphosphate.

2. PGA accumulates when $C^{14}O_2$ is incubated in light with the sonically ruptured chloroplast system in the presence of threose 2, 4-diphosphate. This indicates that PGA is in the major pathway of photosynthetic CO_2 fixation in the system.

3. Threose diphosphate inhibition of the total $C^{14}O_2$ fixation by the carbon cycle of sonically ruptured spinach chloroplasts is not due to inhibition of carboxydismutase, but due primarily to inhibition of triose phosphate dehydrogenase which in turn limits the formation rate of the photosynthetic carbon cycle CO_2 acceptor, RuDP.

THE BIOSYNTHESIS OF OPIUM ALKALOIDS

Frank R. Stermitz and Henry Rapoport

Plants of Papaver somniferum L. were grown in the presence of $C^{14}O_2$ for 2-hr, 6-hr, 8-day periods, after which morphine, codeine, and thebaine were isolated.[1] $C^{14}O_2$ was added only at the start of the experiment and was more than 90% absorbed within 24 hours. The incorporation of radioactivity into each of these alkaloids was determined for (a) the intact compounds, (b) the various O- and N-methyl groups, and (c) the ring skeleton. These results are given in Table VI.

Differences found in the ring-skeleton labeling are best accommodated by a scheme in which thebaine is the precursor of the other morphine alkaloids. Morphine appears to be a storage product formed from codeine by demethylation.

Methylation in biological systems is often considered to be a deactivation process in which active hydrogens (on nitrogen or oxygen) are replaced by inactive methyl groups. The results here indicate that such a conception is certainly not true in regard to the morphine-type alkaloids and therefore cast some doubt on its validity as a general concept.

[1] Frank R. Stermitz and Henry Rapoport, in Bio-Organic Chemistry Quarterly Report, UCRL-8848, June 1959, p. 52.

Table VI

Comparison of specific activities (10^3 dpm/μmole) of peripheral methyl groups and ring carbons

Run No.	Compound	Total	N-Methyl	O^3-Methyl	O^6-Methyl	Ring carbons
9 (2 hr)	Morphine	10.6	5.8[a]	-	-	4.8
	Codeine	197	48.2[b]	51.8[c]	-	77
	Thebaine	2810	340[d]	343[e]	1830[f]	300[d]
10 (6 hr)	Morphine	202	36[a]	-	-	166
	Codeine	4040	1240[b]	876[c]	-	1920
	Thebaine	16500	398[g]	2020[e]	11500[f]	2580
7 (8 days)	Morphine	505	42[a]	-	-	463
	Codeine	575	140[a]	89[c]	-	346
	Thebaine	667	166[g]	132[e]	169[f]	200

[a] Normorphine counted.

[b] Norcodeine counted.

[c] Morphine counted.

[d] Maximum estimation for N-methyl giving minimum estimation for ring carbons.

[e] Dihydromorphine counted.

[f] Dihydrocodeine counted.

[g] Dihydronorcodeine counted.

INVESTIGATION OF ORGANIC MATTER IN METEORITE MURRAY

Susan K. Vaughn and Melvin Calvin

Preliminary investigation of meteorite Murray was reported earlier.[1] More recent data on the carbonaceous material can now be reported. Summaries of two extraction series are presented in Tables VII and VIII. An infrared study of the carbon tetrachloride extract (Table VII) was made on the Beckman IR-7 spectrophotometer. By comparing the methyl and methylene peaks in the 2900-cm^{-1} region of hexane, a C_{27} n-paraffin, and the extract, it was determined that the hydrocarbon in the extract was intermediate between the two, perhaps C_{19}. Figure 5 shows the complete infrared spectrum of the 10.4 mg CCl_4 extract (Table VIII) evaporated on a NaCl plate. The 2900-cm^{-1} region shows methyl and methylene bands; the 1700-cm^{-1} region, which is more highly resolved in Fig. 6, corresponds to carbonyl bonds; the absorption at 1400 to 1500 cm^{-1} is due to methyl and methylene groups; and the absorption at 800 cm^{-1} is due to aromatic hydrogens. The fluorescence spectra of the CCl_4 extract are shown in Fig. 7. No further interpretation of these spectra can be made at this time.

Two small portions of the 500 mg water extract (Table VII) were submitted to a ninhydrin test for amino acids and a silver nitrate test for reducing material. The ninhydrin test was negative, but the silver nitrate test indicated that something was present that reduced silver.

Approximately 150 mg of the water extract was applied to a Dowex-1 column and eluted with 0.03 N HCl. About 5 mg of ultraviolet-absorbing material was eluted off, reapplied to another Dowex-1 column, and eluted gradiently with HCl. Figure 8 shows the effect of this fractionation procedure on the spectrum of the water extract. The last fractionation apparently gave two components whose wave-length maxima differed by about 50 A. Figures 9, 10, and 11 show the effect of pH on the spectra of the fractionated water extract samples. Figure 11 was made on a diluted sample of Water Extract II. These spectra indicate that the pH change is quite reversible.

The ultraviolet spectrum of the CCl_4 extract of the meteorite is included in those shown in Fig. 12. The absorption in the region 290 to 250 mμ is due to elemental sulfur.[2]

The following conclusion was drawn from this work by Dr. Calvin (in a seminar delivered at the Massachusetts Institute of Technology, Cambridge, Massachusetts, November 13, 1959, in connection with the Karl Taylor Compton lectureship): The spectra indicate that an aromatic-type heterocyclic molecule with a pK value somewhere between 4 and 7 is present. The spectrum and behavior of this chromophore are similar to those of cytosine although there is by no means enough evidence for a definite identification.

[1] Susan K. Vaughn, in Bio-Organic Chemistry Quarterly Report, UCRL-8961, June 1959, p. 44.

[2] J. E. Baer and M. Carmack, J. A. Chem. Soc. 71, 1215 (1949).

Table VII

Weight of material extracted by each solvent from a 9-gram sample of meteorite Murray

Solvent	Weight (mg)
1. CCl_4	20.1
2. $CHCl_3$	3.3
3. Benzene	3.9
4. Water	541.1, containing 8% carbon
5. Ethanol	9.3
6. Benzene	0.0

Table VIII

Data on solvent extraction of 4.9-gram sample of meteorite Murray

Solvent	Wt. of material extracted (mg)	Analysis of extract	Weight of meteorite after extraction	Analysis of residual meteorite
CCl_4	10.4		4.9 g	
6N HCl				
48% HF	150	1.6% C	330 mg	18% C
		2.9% H		2% H
		21.8% residue		
CCl_4	35	16.5% C	260 mg	
		3.4% H		
		0.2% N		
		14.2% residue		
H_2O	18		223 mg	24.4% C
				2.0% H

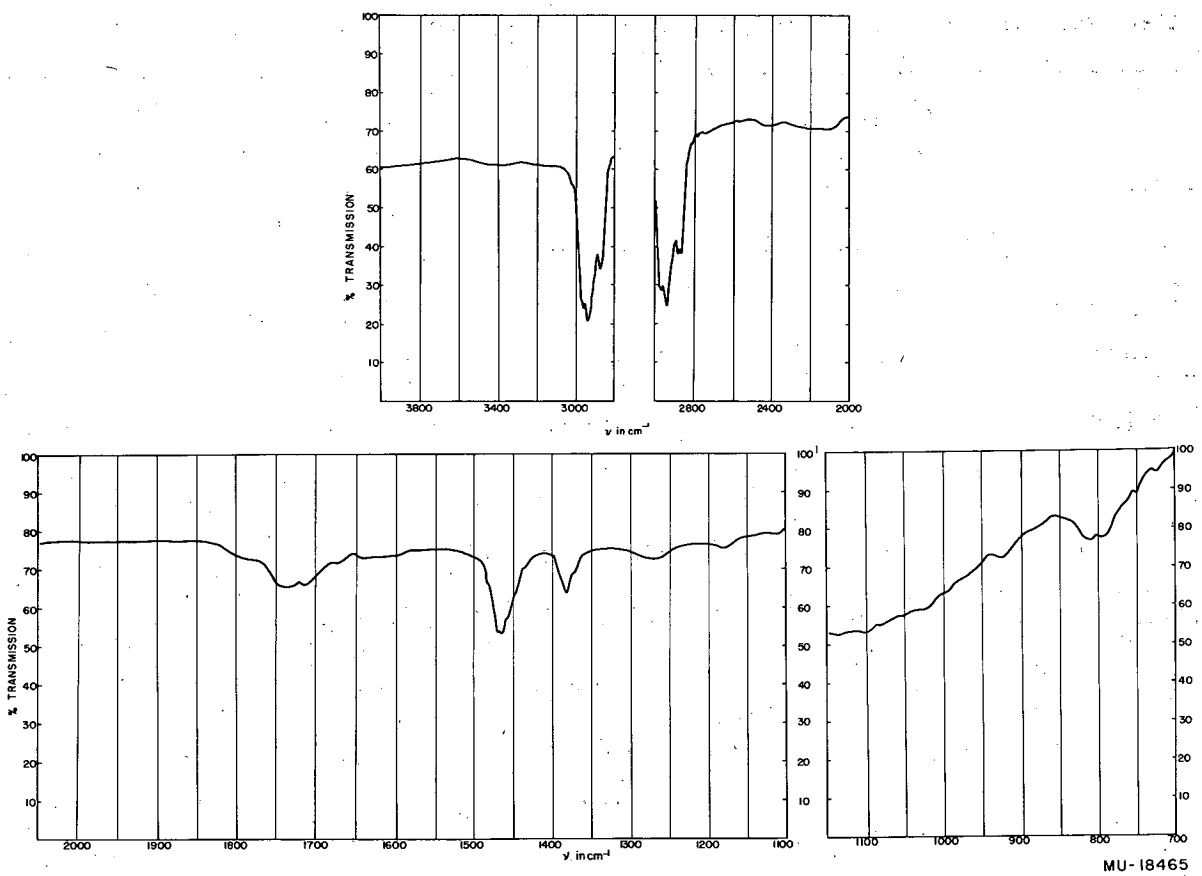


Fig. 5. Infrared spectrum of CCl_4 extract.

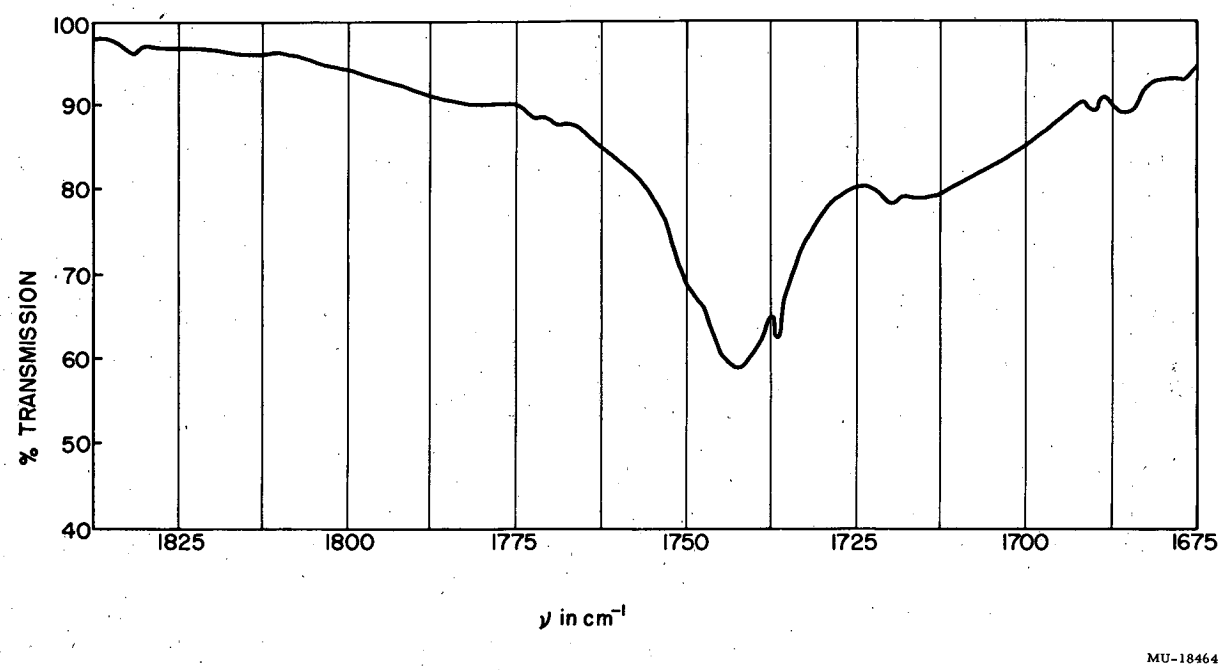


Fig. 6. Highly resolved infrared spectrum of CCl_4 extract.

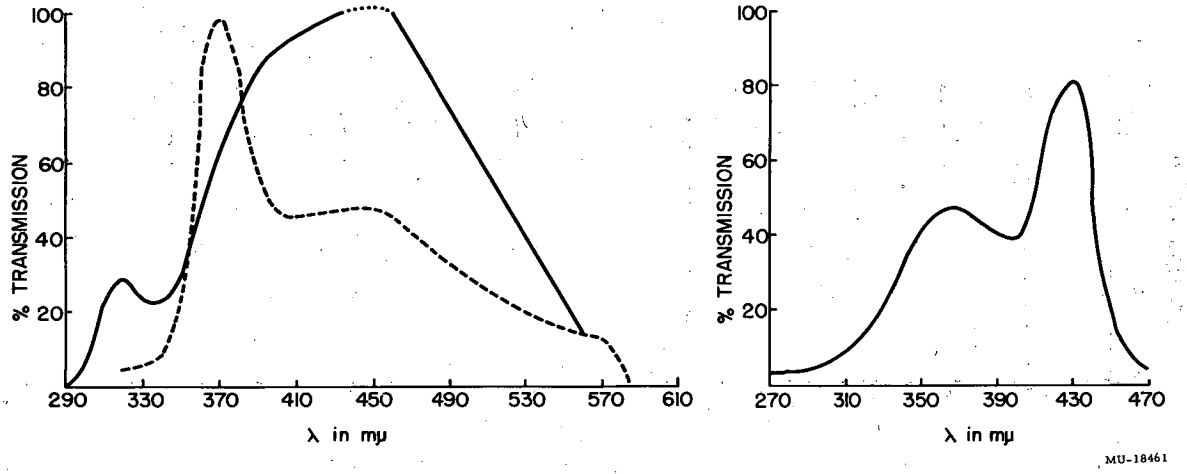


Fig. 7. Left: Fluorescence spectra of CCl_4 extract.
---activation λ = 370 mμ, conc. = 2.3 mg/ml.
——activation λ = 310 mμ, conc. = 690 mg/ml.
Right: Activation-wavelength spectrum (Fluorescence λ = 430 mμ, conc. = 2.3 mg/ml.

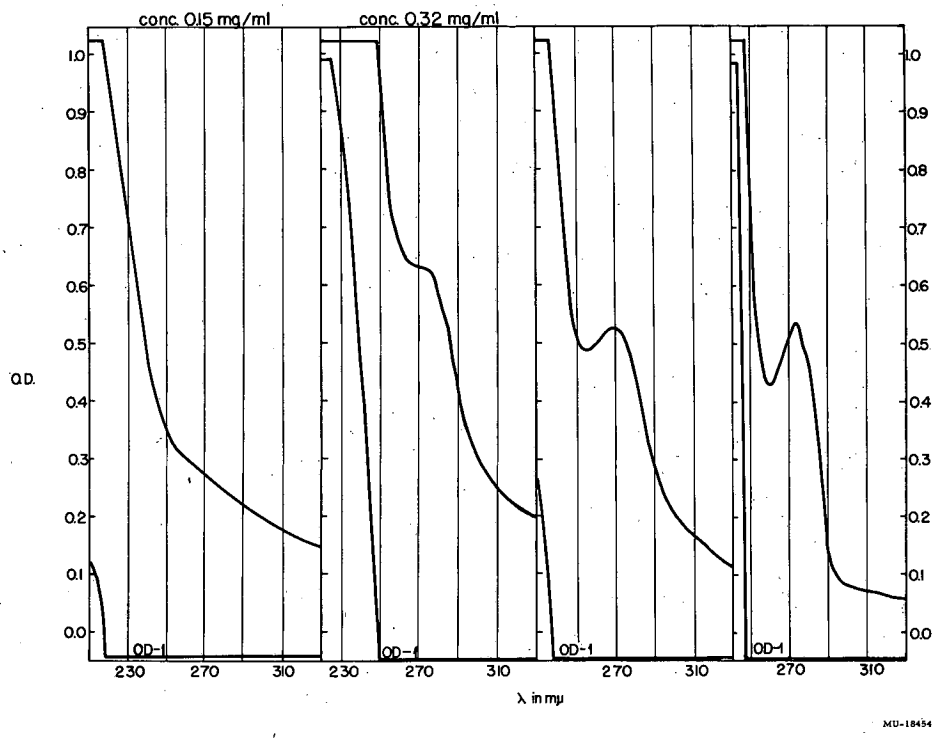


Fig. 8. Fractionation of H_2O extract from Dowex-1 column. Left, original H_2O extract; left center, after first fractionation; right center and right after second fractionation.

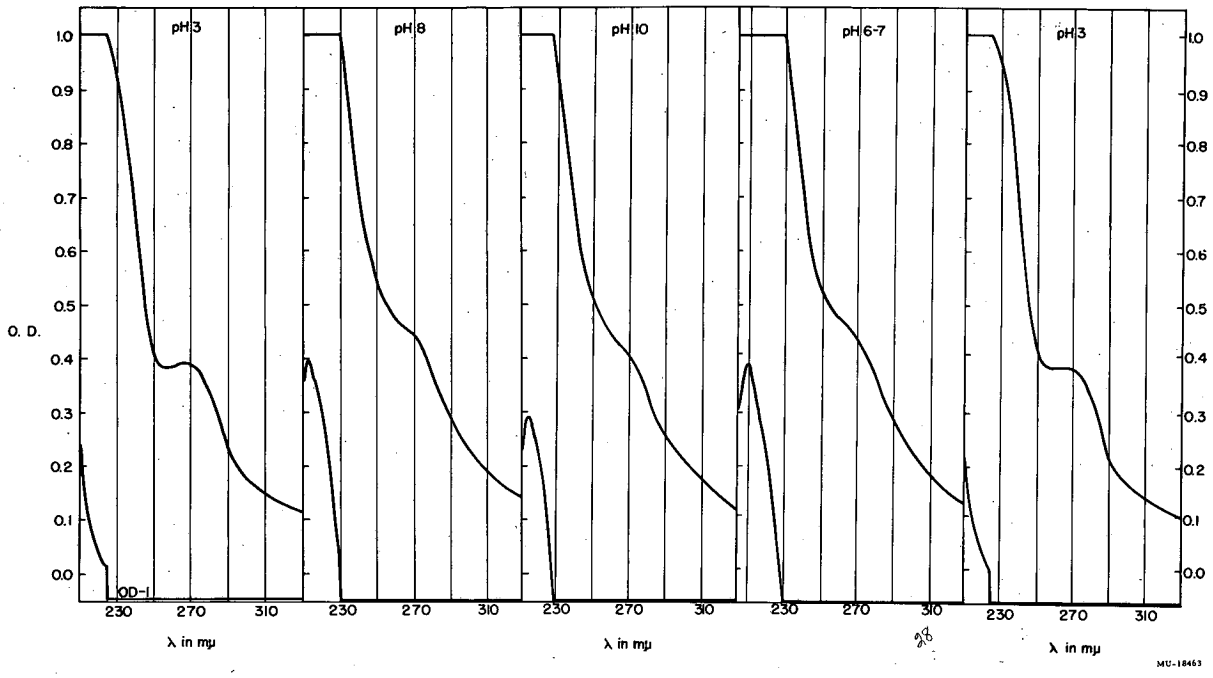


Fig. 9. Effect of pH on ultraviolet spectrum of water Extract I.

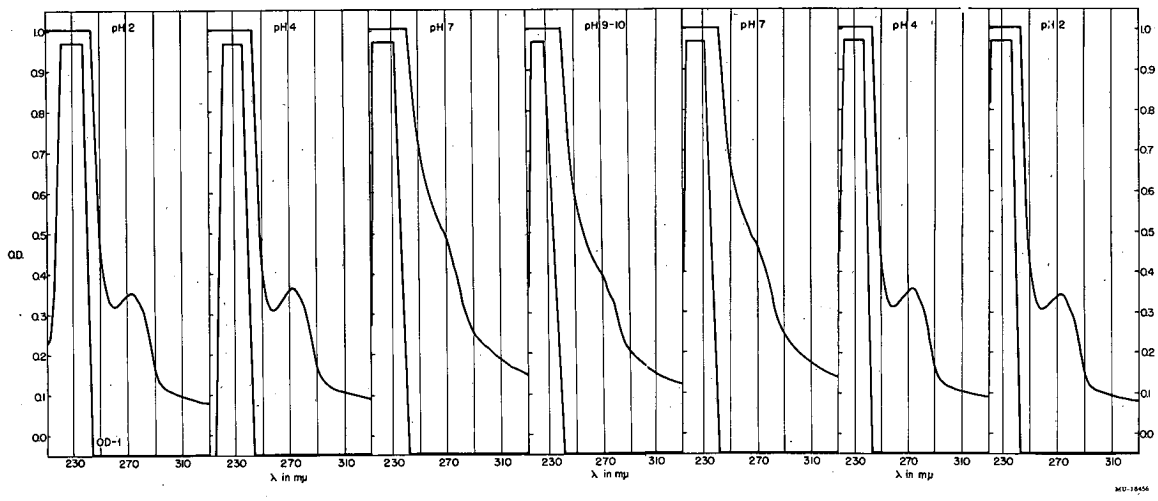


Fig. 10. Effect of pH on ultraviolet spectrum of water Extract II.

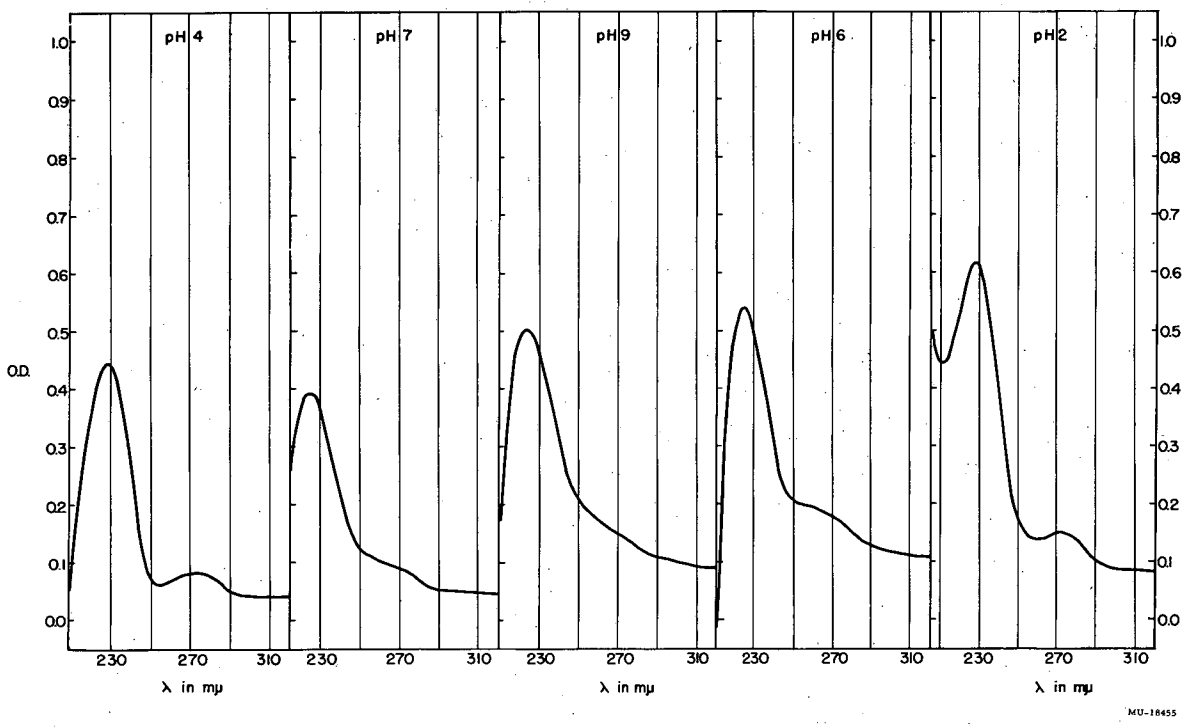


Fig. 11. Effect of pH on ultraviolet spectrum of diluted sample of water Extract II.

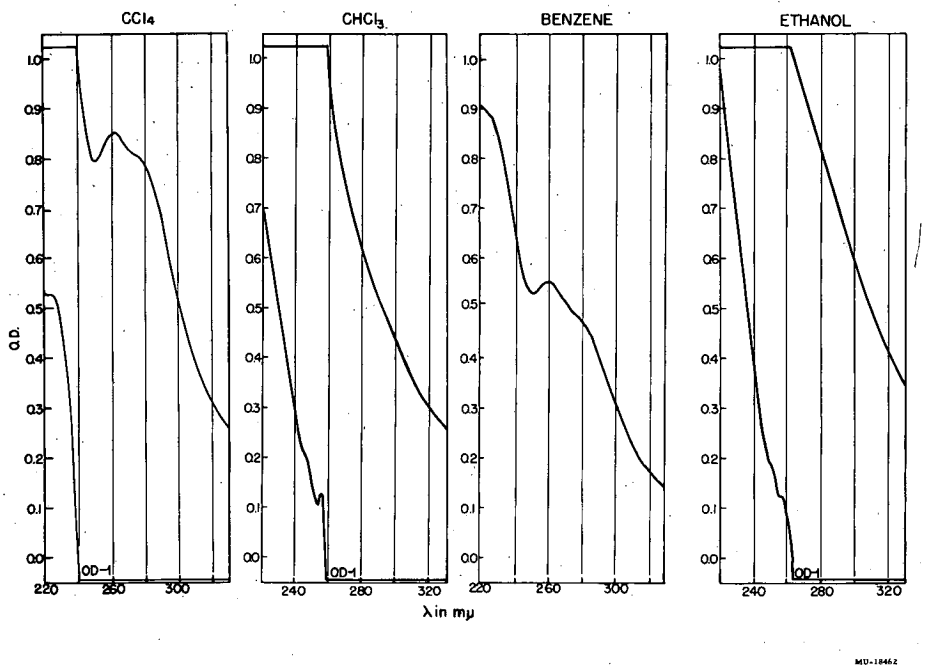


Fig. 12. Ultraviolet spectra of various extracts of meteorite (solvent: methylcyclohexane; concentration: 0.15 mg/ml).

STRAIN DIFFERENCES IN ACETYL CHOLINE CONCENTRATION IN RAT BRAIN

Edward L. Bennett, Marie Hebert, Ann Ohlander, and Patricia Smith
(In collaboration with Professor David Krech and Mark R. Rosenzweig, Department of Psychology, University of California, and James Crossland, Department of Physiology, St. Andrews University, St. Andrews, Scotland)

In numerous experiments we have found correlations between cortical cholinesterase (ChE) activity and maze behavior when the S_1 and S_3 strains of rats were tested.[1] These correlations were found on an individual basis within a rat strain as well as between strains. Those animals which had high cortical ChE activity (expressed as the average activity of the visual and somethetic areas or $\frac{V + S}{2}$) generally made fewer errors in the several maze tests used. The level of ChE activity was assumed to afford an index of metabolism of the acetyl choline (AcCh)-ChE system. To test the generality of a positive correlation between cortical ChE activity and maze behavior, rats from two foundation stocks were selectively bred for high and low ChE activity.[2,3] Although these animals differ even more than the S_1 and S_3 animals in ChE activity, only small behavioral differences have been found. Moreover, contrary to the findings with the S_1 and S_3 strains, animals of the new low-ChE strains (RDL and RCL) tended to make fewer, not more, errors in various maze tests than did animals of the new high-ChE strains (RDH and RCH).

In an attempt to encompass the two sets of apparently contradictory findings, we revised our former hypothesis.[1] We now made the assumption that the level of ChE activity was not necessarily correlated among all strains of rats with the level of AcCh metabolism. We then suggested that the important measure might be a function of both AcCh (or choline acetylase) and ChE activity.[1] This suggestion is based on the consideration that, at certain synapses, transmission is determined by the total action of released acetyl choline over time. Our revised hypothesis assumed that between the S_1 and S_3 strains (which had been bred for maze-bright and maze-dull performance, respectively), there would be a significant difference in both AcCh and ChE activity. The S_1 strain would both release larger amounts of AcCh at synapses and have more ChE to hydrolyze it than the S_3 strain. On the other hand, the animals selected for high and low ChE activity might

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[1] E. L. Bennett, H. Roderick, A. Ohlander, H. Morimoto, C. Longueil, D. Krech, and M. Rosenzweig in Chemistry Division Quarterly Report, UCRL-8575, Dec. 15, 1958, p. 34.

[2] T. H. Roderick, E. L. Bennett, and H. Karlsson in Chemistry Division Quarterly Report, UCRL-3950, Sept. 1957, p. 86.

[3] E. L. Bennett, H. Karlsson, N. Dye, A. Ohlander, and T. H. Roderick, in Chemistry Division Quarterly Report, UCRL-8421, June 1958, p. 44.

not differ in availability of AcCh. Thus, for these strains, high ChE activity might mean inadequate AcCh action, and these strains might show poorer maze behavior. The important measure for determining maze performance may be the ratio of AcCh or of choline acetylase (the enzyme synthesizing AcCh) to ChE activity. We therefore predicted that the S_1 and S_3 animals would differ in AcCh concentration whereas the RH and RL animals would not differ in AcCh concentration. This suggestion has been confirmed by the experiments described in more detail below.

Extraction and Assay of Acetyl Choline

AcCh is a very labile constituent of brain. Therefore the technique used for killing an animal and extracting the AcCh must eliminate or minimize changes in the "normal" AcCh content.[4] The brain was frozen in situ by immersing the rat in a small cage into a large Dewar containing liquid oxygen.[5] The brain (excluding cerebellum) was chipped out of the frozen head, weighed rapidly, and crushed in a stainless-steel crusher.[6,7] The frozen, powdered brain was immediately transferred to a mortar (which had been chilled on a block of dry ice) containing 95% ethanol with 0.2% glacial acetic acid.[8,9] Five ml of ethanol was used for each gram of brain. The brain was ground with the alcohol over a 10-minute period and gradually allowed to warm to about 0°. It was centrifuged and washed twice with 2.5 ml of 70% ethanol with 0.15% glacial acetic acid. Five ml of water was added to the combined extracts in a 40-ml centrifuge tube and the extract was concentrated to 1 to 2 ml by an air stream at room temperature. This required 12 to 18 hours. The solution was transferred to recalibrated 12-ml centrifuge tubes using frog Ringer solution[5] to rinse the 40-ml tube. The pH was adjusted to 4.0 with 0.5 N NaOH, and the solution was diluted to 8.0 ml with frog Ringer solution. The solutions were stored in a deep freeze until analysis.

For analysis, the extract was divided into two equal portions; one half was adjusted to pH 6.0 and diluted to 6.0 ml, the other half was adjusted to pH 12 with 4 drops of 1 N NaOH, heated in a boiling water bath for 10 minutes, cooled, neutralized to pH 6.0, and diluted to 6.0 ml. The AcCh was determined in the first tube, the second tube served as a "blank," and known amounts of AcCh were added to it to serve as standards and control for sensitizing substances which occur in variable amounts in brain.[10]

[4] Dr. James Crossland, St. Andrews University, gave us valuable assistance and instruction in the AcCh determination.

[5] J. Crossland, J. Physiol. 114, 318 (1951).

[6] J. Crossland, H. M. Pappius, and K. A. C. Elliott, Am. J. Physiol. 183 27 (1955).

[7] W. E. Stone, Biochem. J. 32, 1908 (1938).

[8] W. E. Stone, Arch. Biochem. Biophys. 59, 181 (1955).

[9] Preliminary experiments indicated that with rat brain frozen in situ, about 15% more AcCh was found when the acidulated-alcohol-extraction method of Stone was used than when 10% trichloroacetic acid was used as described by Crossland.[5]

[10] W. Feldberg, J. Physiol. 114, 318 (1951).

The frog (Rana pipiens) rectus abdominis preparations were used to determine the AcCh content of the extracts.[11] Neostigmine bromide (2.5 μg/ml) was used to sensitize the muscle.[6] A transducer (Statham Instrument Co. Model 987-G7A, ±0.15 oz) and a Leeds and Northrup Speedomax recorder with 4-mv full-scale sensitivity were used to measure the isometric contraction. A 1-gram increase in tension resulted in full-scale deflection of the recorder. The muscle was prestretched at 0.75 g tension, and 0.5 g tension was maintained during assay.

To determine the AcCh content of a sample, the contractions obtained in 90 seconds with alternate aliquot portions of the sample were compared with those obtained from the blank containing known amounts of added acetyl choline perchlorate. The muscle bath contained 4.5 ml. In general, the amount of AcCh in an extract was determined by finding known amounts of AcCh (usually differing by about 10%) that produced contractions slightly less and slightly more than given by the unknown (a flanking or bracketing procedure).

The sensitivity of a muscle preparation varies, both daily and during the day; generally, 150 to 400 μl of the brain extract was taken for each contraction. Since a rat brain contains approximately 30 mμmoles of AcCh, a measurable contraction required the equivalent of 0.07 to 0.18 μg of acetyl choline chloride. Assays were made at fixed intervals, usually 5 minutes.

All samples were coded and the analysis order was randomized to avoid bias or systematic errors. Duplicate analyses were made of all samples by different analysts. About 80% of the analyses agreed within 10%, and few differed by more than 15%. When an analysis differed by more than 10%, a third analysis was made.

The results of the AcCh determinations are summarized in Table IX. The S_1 and S_3 rats, descendants from animals selected about 30 years ago on a behavioral basis, differ significantly (approximately 13%) in AcCh concentration in the brain. S_1 rats had a higher AcCh concentration than any of the other five strains tested. The RDL and RDH rats, although differing greatly (about 15%) in ChE activity, do not differ in AcCh concentration in the brain. The RCL and RCH animals, also differing about 15% in ChE activity, differ by about 9% in brain AcCh content. The results suggest that the genetic factors controlling AcCh concentration and ChE activity in the brain are different.

The difference in ChE activity of these strains can only be estimated at present for comparable samples. For the AcCh determination, the whole brain, including cortex but excluding the cerebellum, was used. The ChE data have been obtained for brain including cerebellum and not including the cortex. ChE data have also been obtained for the visual and somesthetic areas of the cortex. When additional rats of these strains are available, we will determine ChE activity for a sample comparable to that used for the AcCh determinations in order that the results can be

[11] F. C. MacIntosh and W. L. M. Perry, Methods in Medical Research (Year Book Publishers, Chicago, 1950) v. 3, p. 78.

Table IX

Brain AcCh concentration of six strains of rats

Strain	Number of rats	Age	Brain Weight (g) ± $\bar{\sigma}$	AcCh/g (mμM) ± $\bar{\sigma}$	Total AcCh (mμM) ± $\bar{\sigma}$
S_1	16	125-151	1.196 ± 0.096	27.21 ± 2.00	32.42 ± 2.09
S_3	19	134-165	1.298 ± 0.107	24.07 ± 2.32	31.15 ± 3.03
$\Delta (S_1-S_3)$			-0.102	3.14	1.27
t			2.85	4.13	1.37
P			<0.01	<0.01	
RCH	11	144-152	1.464 ± 0.073	24.52 ± 1.57	35.89 ± 3.01
RCL	11	144-159	1.523 ± 0.070	22.47 ± 2.70	34.16 ± 3.81
Δ (RCH-RCL)			-0.059	2.05	1.73
t			1.85	2.07	1.13
P				0.05	
RDH	14	150-179	1.280 ± 0.102	23.13 ± 1.48	29.53 ± 1.97
RDL	16	150-177	1.426 ± 0.086	22.98 ± 1.96	32.70 ± 2.54
Δ (RDH-RDL)			-0.146	0.15	-3.17
t			4.10	0.22	3.67
P			<0.01		<0.01

$$\bar{\sigma} = \sqrt{\frac{\Sigma x^2}{N-1}}, \qquad t = \frac{\bar{X}_1 - \bar{X}_2}{\bar{\sigma}_{\bar{x}_1 - \bar{x}_2}}$$

$$\sigma_{\bar{x}_1 - \bar{x}_2} = \sqrt{\frac{(N_1 + N_2)(\Sigma x_1^2 + \Sigma x_2^2)}{N_1 N_2 (N_1 + N_2 - 2)}}$$

expressed as a ratio of AcCh concentration to ChE activity. In addition, we plan to determine choline acetylase activity in these strains of rats. No data are available on correlation between choline acetylase activity and ChE activity or AcCh concentration. The ratio

choline acetylase activity/ChE activity

may be an even better indicator of neuronal efficiency.

UNPAIRED ELECTRONS IN SYSTEMS BOUND BY CHARGE-TRANSFER FORCES

John W. Eastman and M. Calvin

I. AROMATIC HYDROCARBONS

1. Charge-Transfer Spectra

The presence of charge-transfer binding may be recognized by the deep color that results when solutions are prepared from combinations of good electron donors and acceptors. This charge-transfer absorption occurs at 825 mμ in a perylene-o-chloranil (tetrachloro-1,2-benzoquinone) solution, and at 745 mμ in solutions of perylene-chloranil (tetrachloro-1,4-benzoquinone).

2. Concentration of Unpaired Electrons

Previously it was shown that electron spin resonance (ESR) absorption occurred when o-chloranil was combined with perylene in the crystalline 1:1 complex.[1] The number of unpaired electron spins observed depends upon the method of preparation and upon how long the sample is exposed to air.

The number of unpaired electrons was estimated by comparing the observed peak-to-peak height of the derivative of the ESR absorption of the sample with that height for a known number of solid diphenyl-picryl-hydrazyl (DPPH) radicals. The differences noted are good within ± 50% ; the absolute values are correct to an order of magnitude.

The mole fractions of unpaired electrons are different by factors of several hundreds for different preparations. An example is given in Table X. These values were obtained within 24 hours after preparation.

The chemical analyses were used to calculate the ratios of the number of donor molecules to the number of acceptor molecules for one preparation (Table X). The solid was prepared by dissolving the components (one perylene for every o-chloranil) in boiling CCl_4 in air, cooling, filtering, and vacuum-drying the precipitate. The analyses and ESR absorption were determined for this solid (Table X, column 2) and also for a recrystallized sample (column 3). The latter was prepared by dissolving the original precipitate in boiling CCl_4 in air, filtering the hot solution, cooling slowly (6°/hr), filtering, and vacuum-drying. When the solid was prepared in heptane and $CHCl_3$ the analyses were somewhat more different from that predicted for a 1:1 complex and the number of unpaired electrons was much greater. When crystallization from $CHCl_3$ was attempted by slow cooling over a period of days a brown amorphous-appearing solid resulted.

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[1] J. W. Eastman and M. Calvin, in Bio-Organic Chemistry Quarterly Report, UCRL-8575, Dec. 1958, p. 17.

Table X

Ratio of the number of perylene molecules to the number of chloranil or o-chloranil molecules; also numbers of unpaired electrons.

Based upon Analysis of	perylene-o-chloranil		perylene-chloranil
	Original crystallization	After recrystallization	
Carbon	0.953	0.976	0.930
Hydrogen	1.01	0.978	1.23
Chlorine	1.01	1.03	0.959
mol fraction of unpaired electron spins (within 24 hours after preparation)	3×10^{-7}	4×10^{-8}	3×10^{-8}

The solid perylene-chloranil was prepared by dissolving the components (one perylene for every chloranil) in boiling $CHCl_3$ in air, cooling, partially evaporating, filtering, redissolving in boiling CCl_4, filtering the hot solution, partially evaporating, filtering, and vacuum drying. For perylene-chloranil the donor-acceptor ratio differs from 1:1 more than for perylene-o-chloranil.

Fresh preparations of the perylene-quinone solid adducts contain approximately 1×10^{-8} mol fraction unpaired electron spins.

Observation of the ESR absorption in solutions of these adducts is limited by their solubility. If the number of unpaired electrons per complex remained the same in solution and the solubility were approximately the same as that of the separate components, no absorption could be seen. In fact, some absorption does occur for solutions in CCl_4, but it is very small.

3. Time Dependence of the Number of Unpaired Electrons

The recrystallized solid of perylene-o-chloranil was divided into two samples. One was kept in vacuo. The other was left in contact with the atmosphere. The number of unpaired electron spins in the evacuated sample increased by a factor of ten in 10 days. The sample in air exhibited a decrease by the same factor (Fig. 13). In addition, the line width increased, which complicated the determination, but a distinct difference was observed.

The unevacuated perylene-chloranil solid exhibited an increase in absorption with time.

The chemical and physical causes of this change are not known. They probably are a combination of chemical reactivity and crystal forces and imperfections.

II. ARYLAMINES

It was previously shown that there were unpaired electron spins when chloranil was dissolved in dimethyl aniline.[1] These unpaired electrons are a chemical intermediate and their concentration depends upon the time of reaction (Fig. 14).

Two experiments under similar conditions resulted in the same concentration dependence (Experiments 1 and 2). A saturated solution of chloranil in dimethyl aniline was reacted in an atmosphere of reduced oxygen content at room temperature. In a third experiment the materials were more carefully purified, oxygen was more carefully excluded, and the concentration of chloranil was reduced. The number of unpaired electrons increased at a much slower rate (Figs. 14 and 15). In Experiment 3 the peak concentration of unpaired electrons was ten times that in Experiments 1 and 2. However, the final concentration was approximately 1×10^{-6} molar in both cases. The products and intermediates of the reaction are being studied.

A charge-transfer complex forms instantaneously on dissolution of chloranil in dimethyl aniline and decays as the reaction proceeds. This complex itself contains no unpaired electron spins.

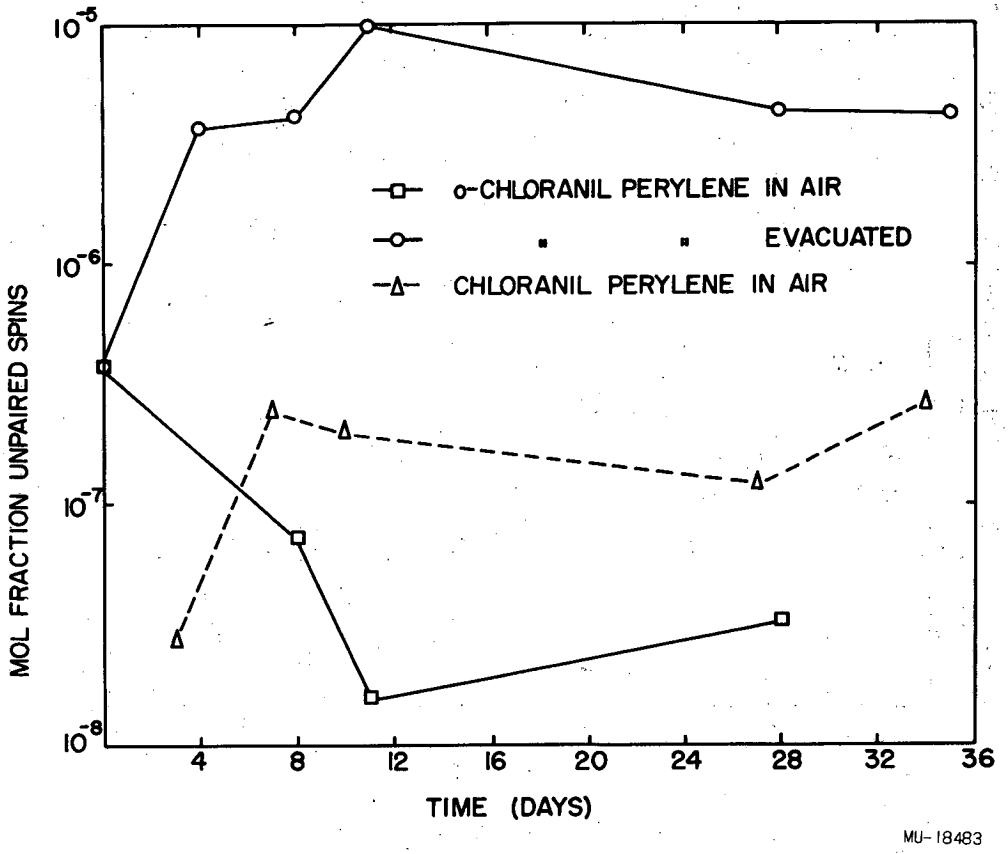

Fig. 13. The time dependence of the number of unpaired spins in the charge-transfer bound solids: perylene-o-chloranil and perylene-chloranil.

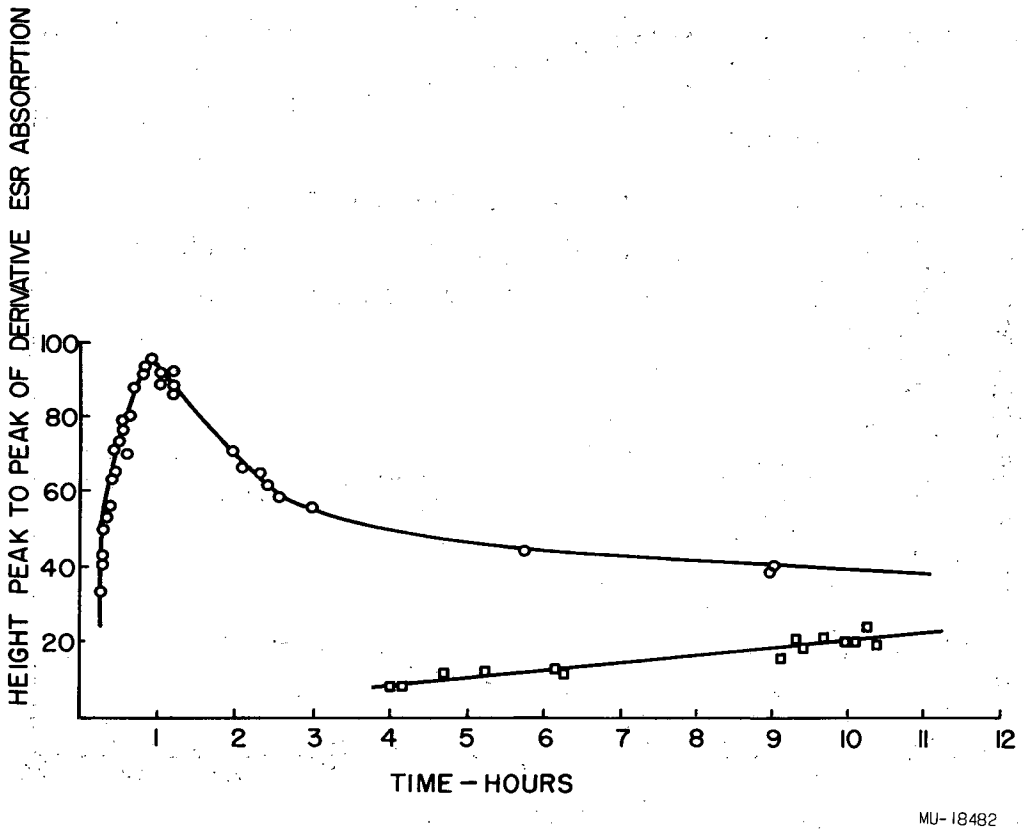

Fig. 14. Time dependence of the formation of unpaired electron spins in a dimethyl aniline solution of chloranil.
-0- initially 0.4 molal chloranil (saturated). Experiments 1 and 2.
-0- initially 0.144 molal chloranil. Experiment 3.

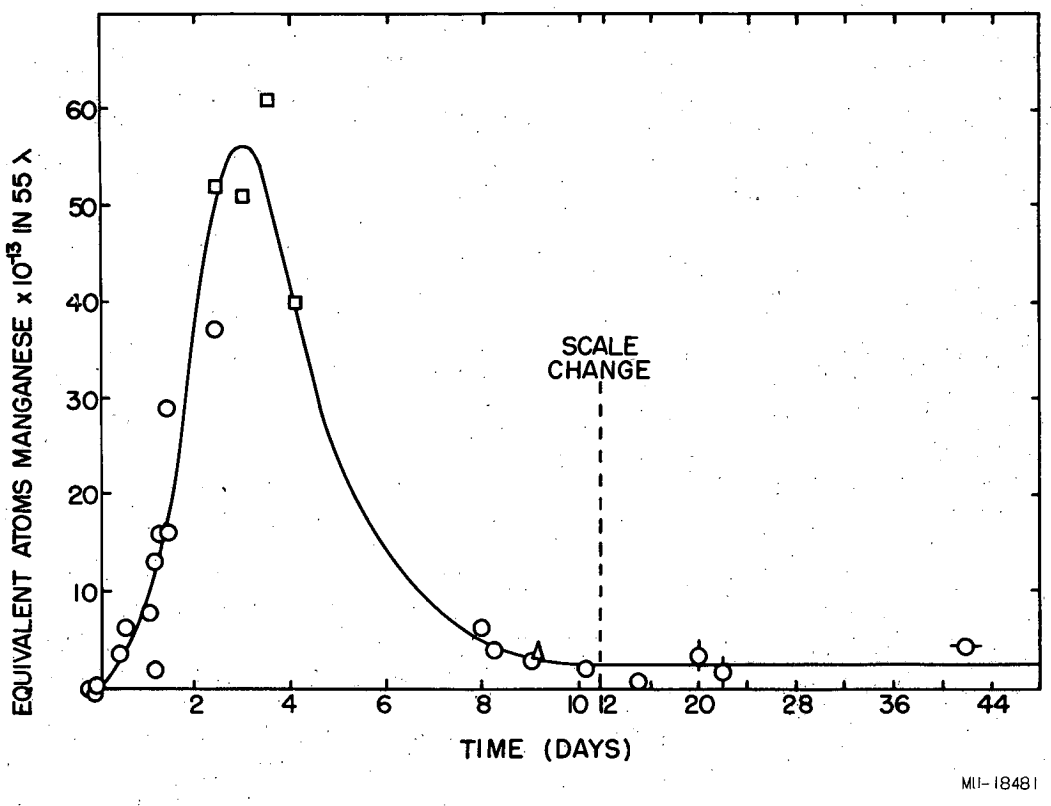

Fig. 15. Time dependence of the formation of unpaired electron spins in a dimethyl aniline solution of chloranil initially 0.144 molal chloranil. Manganese standards containing 5.9×10^{14} atoms (o), 17.9×10^{14} atoms (□), 8.1×10^{14} atoms (Δ), 0.64×10^{14} atoms (φ) and 0.67×10^{14} atoms (θ) were used for calculation (10×10^{13} equivalent atoms of manganese in 55 λ is 2.9×10^{-6} molar manganese).

PROTON MAGNETIC RESONANCE IN SIMPLE POLYPEPTIDES

Tatsumi Ohki

For obtaining the necessary background knowledge for the study of peptides and proteins by proton magnetic resonance, spectra of some simple polypeptides and a related compound have been observed by using a Varian 60-Mc nuclear magnetic resonance (NMR) spectrometer. Although various solvents were used, satisfactory results were obtained only with solutions in water (both H_2O and D_2O) and in formic acid. Solubilities of the polypeptides in other solvents were too small to reveal the fine structures of the spectra. The compounds studied were N-acetylglycine, DL-alanylglycine, glycyl-L-leucine, glycylglycine, glycylglycylglycine, and tetraglycine. In most cases, the absorptions by the protons in various parts of the polypeptides were observed as separate peaks. The absorptions by the hydrogens of the NH_2 and COOH groups were not observed because these protons exchange rapidly with the protons of water. Absorption by NH protons was not observed either, probably because of the spin-spin coupling with the nitrogen nucleus. This interaction may broaden the width of the absorption by the proton so that it may no longer be observed.

Figure 16 shows the proton magnetic resonance spectra of DL-alanylglycine in water, heavy water, and formic acid, respectively. The large peak at the left is the absorption by the proton of water. The CH and CH_3 absorptions are split to four and two peaks, respectively, by the spin-spin coupling between the protons of these two groups. The CH_2 absorption is also split to two peaks in water and acid. This shows that the proton in the NH group does not exchange rapidly with the proton of water. The protons of the NH_2 and COOH group are expected to exchange rapidly with the proton of water so that they will not cause splitting. In D_2O (Fig. 16b) the CH, CH_2, and CH_3 peaks appear in the same positions as in water solution. The only difference is that the CH_2 peak is single. Apparently the NH proton has been exchanged by deuterium, and the spin-spin coupling between the NH and CH_2 protons no longer takes place.

The spectra of N-acetylglycine (Fig. 17) and glycyl-L-leucine (Fig. 18) are almost the same as that of DL-alanylglycine. The absorption by the proton of the CH_3 group of N-acetylglycine appears at a considerably lower frequency than that of DL-alanylglycine. The absorptions by the protons of the CH and CH_2 groups in the side chain of glycyl-L-leucine apparently appear in the same area.

Figures 19, 20, and 21 show the spectra of three compounds: glycylglycine, glycylglycylglycine, and tetraglycine. As usual, the large peak at the left is the absorption by the proton of water. The absorption by the protons of the CH_2 groups of glycylglycylglycine (Fig. 20) consists of four peaks, two of which form a pair. In D_2O, this pair becomes a single peak, as in the CH_2 absorption in DL-alanylglycine. This indicates that the pair of peaks may represent the absorption by the protons of the CH_2 group that is adjacent to the COOH group. It is also expected, as for DL-alanylglycine, that the proton of the NH group (adjacent to the CH_2

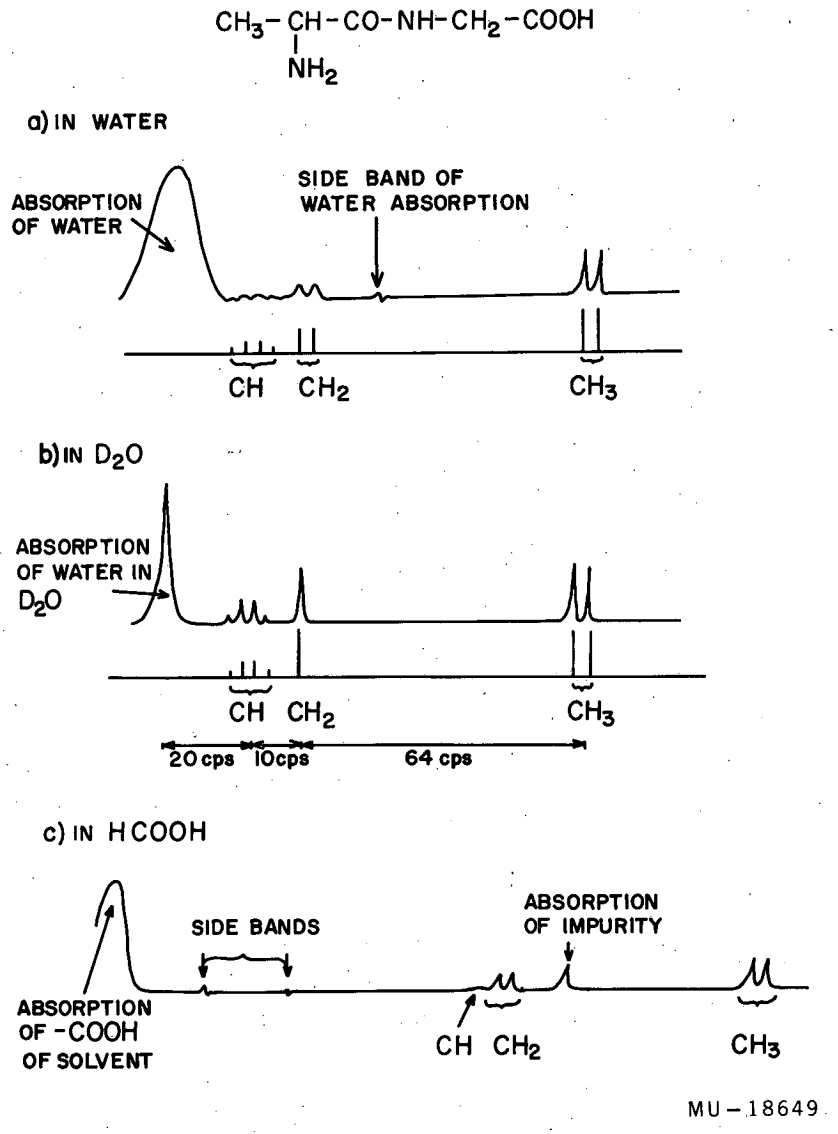

Fig. 16. NMR spectra of DL-alanylglycine in (a) H_2O, (b) D_2O, and (c) HCOOH.

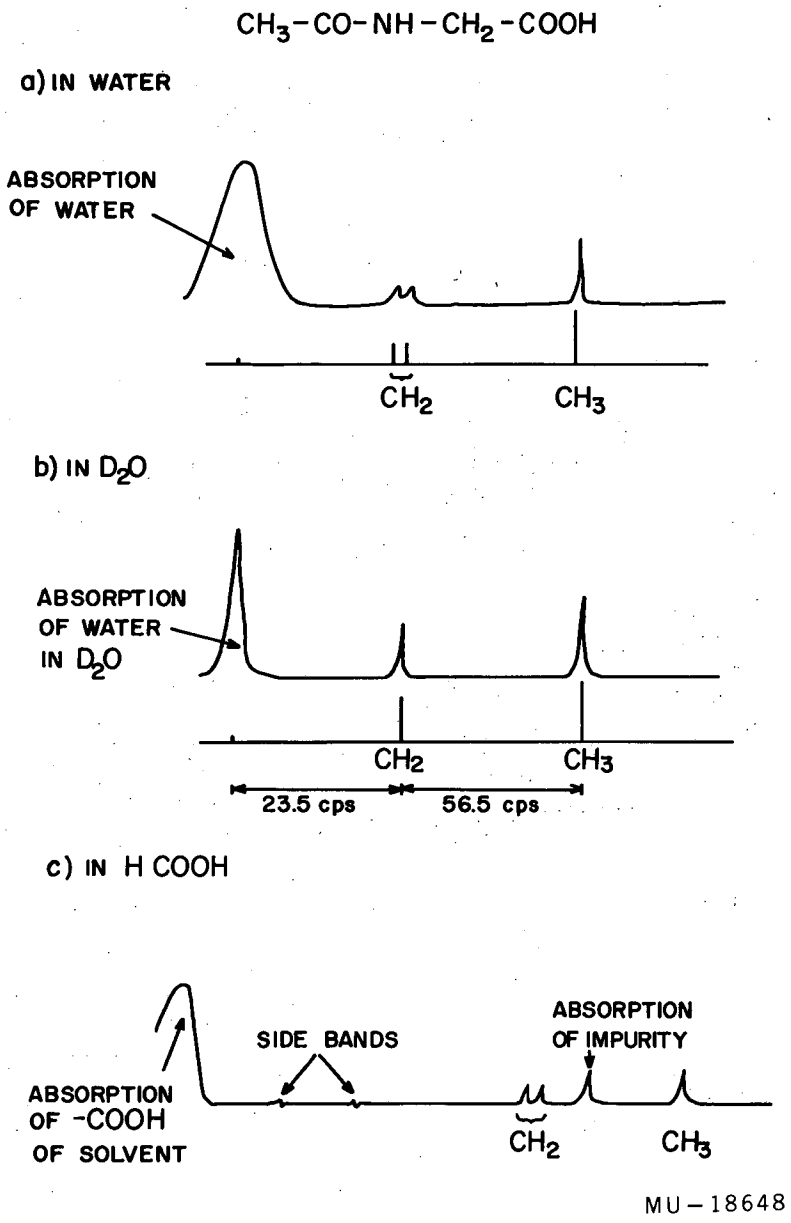

Fig. 17. NMR spectra of N-acetylglycine in (a) H_2O, (b) D_2O, and (c) HCOOH.

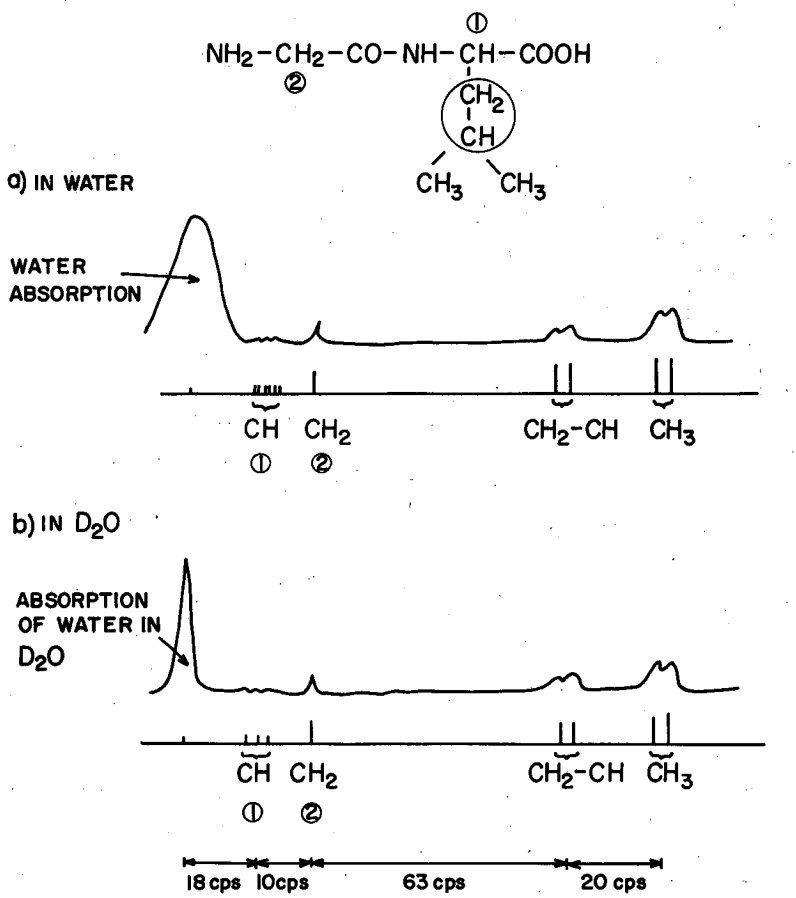

Fig. 18. NMR spectra of glycyl-L-leucine in H_2O and D_2O.

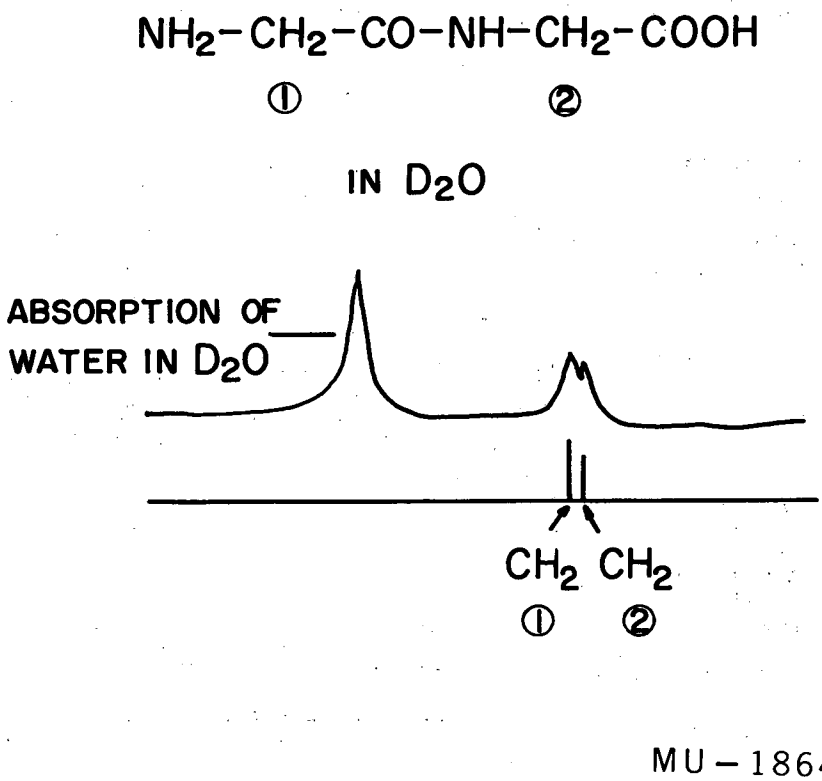

Fig. 19. NMR spectrum of glycylglycine in D_2O.

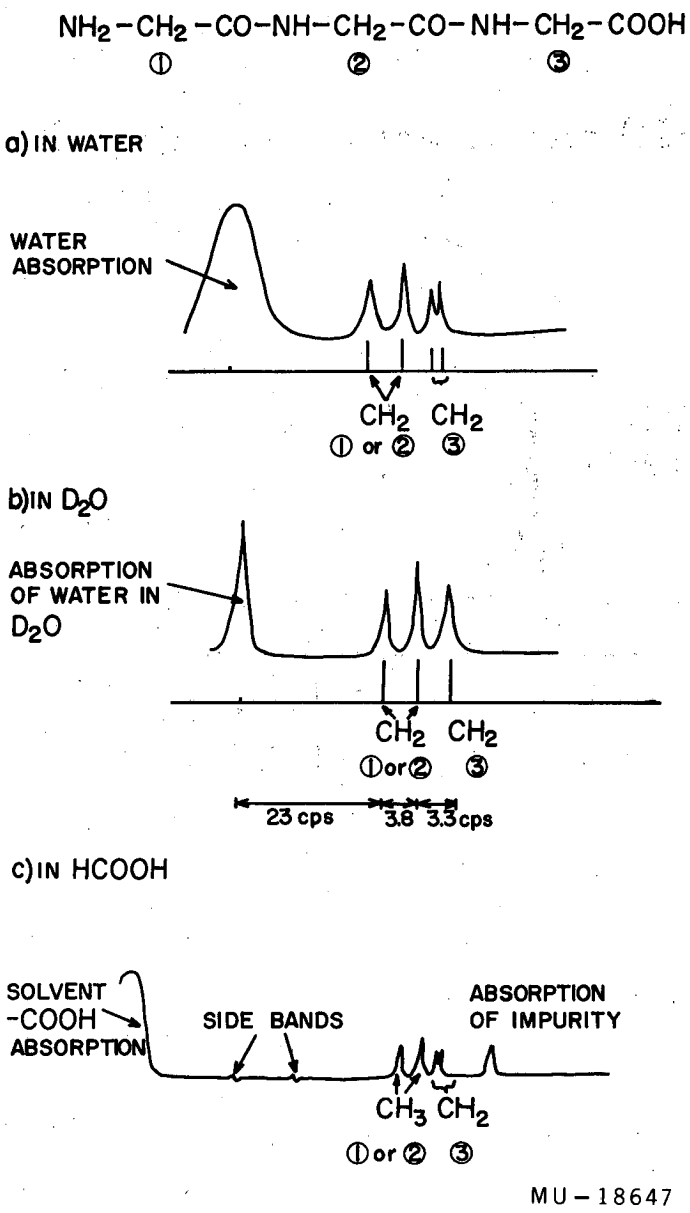

Fig. 20. NMR spectra of glycylglycylglycine in (a) H_2O, (b) D_2O, and (c) HCOOH.

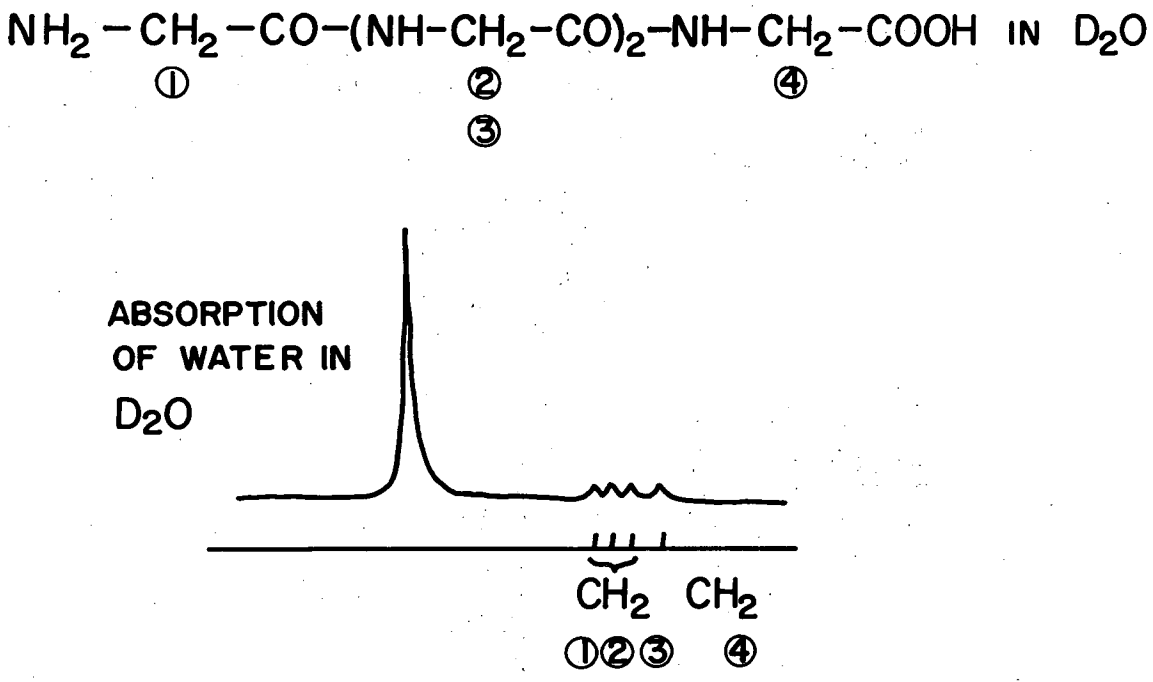

Fig. 21. NMR spectrum of tetraglycine in D_2O.

group under consideration) would not exchange rapidly with the proton of water. The other two CH_2 peaks are interpreted as being the absorption by the protons of the ① and ② CH_2 groups, though it is difficult to show which peak corresponds to which.

For glycylglycine and tetraglycine, the spectra of proton resonance of the CH_2 groups are almost the same as for glycylglycylglycine, but the positions of the peaks are slightly different. It should be noted that the four different CH_2 groups of tetraglycine have different absorptions. Even the CH_2 ② and CH_2 ③ group absorptions are slightly different.

PRELIMINARY STUDIES ON THE OCCURRENCE OF MANGANESE PHAEOPHYTIN IN PLANTS

Edward Markham

The initial stage of the photosynthetic cycle requires the capturing and conversion of electromagnetic energy to electronic potential energy.[1] This occurs through the agency of the chlorophyll pigment system. A conceivable mechanism for such a process may be envisaged as follows: The chlorophyll molecule, through the absorption of light energy, is raised to an excited state, and then returns to the ground state by passing on its excitation energy to a receptor molecule. The actual energy conversion would then be carried out by the receptor molecule. Bourdon considered that iron, copper, or manganese phaeophytins could act in such a manner by virtue of the fact that these metals can exist in two (or more) oxidation levels.[2] He proposed the following scheme:

$$h\nu + P_{Mg}\,II \rightarrow P_{Mg}\,II^{*} \ ,$$

$$P_{Mg}{}^{*}II + P_{Fe}II \rightarrow P_{Mg}II + P_{Fe}{}^{*}II,$$

$$P_{Fe}{}^{*}II \rightarrow P_{Fe}III + e^{-}$$

(where P = phaeophytin).
The excited chlorophyll passes on its energy to the iron phaeophytin in the ferrous state, which then evolves an excited electron. The ferric phaeophytin is then reduced back to the ferrous state by the abstraction of an electron from a molecule of water while the excited electron is transferred to an electron acceptor. Such a receptor molecule need be present only in trace amounts. Bourdon's results did in fact show that an iron phaeophytin was present. We decided to reinvestigate this problem and consider the possibility of manganese phaeophytin as a receptor molecule also. The use of electron spin resonance (ESR) suggested itself as a sensitive method of detecting manganese. In order to undertake this work it has been found necessary to prepare model compounds of manganese to study their ESR and to try to relate it to their structure.

Three chelates of manganese have been prepared, manganese phthalocyanine (Fig. A), pyridine manganese (IV)-phthalocyanine oxide (Fig. B), and bis-(3:3':4, 4':5, 5''-hexamethyldipyrromethene)-manganese (II) (Fig. C).

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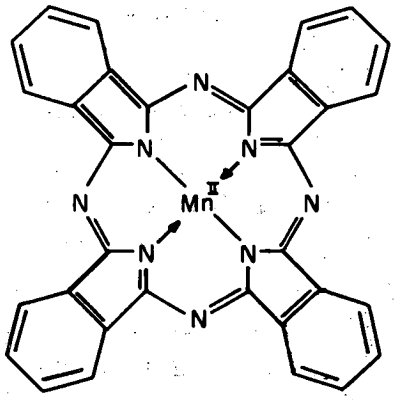
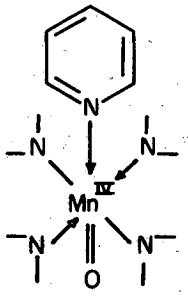
[1] James A. Bassham and M. Calvin, Photosynthesis, UCRL-2853, Feb. 1955.

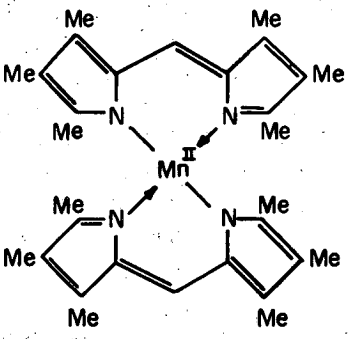
[2] J. Bourdon, Iron Chlorophyll Compounds in Green Plants, unpublished final report, September 1956.



A



B



C

MU-19010

Manganese phthalocyanine (A) was prepared by the method of Barret, Dent, and Linstead[3] and then further purified by sublimation at 400° at 0.5 cm Hg. It was obtained as long black needles which on treatment with pyridine rapidly gave a blue solution. The molecular species responsible for this color has been attributed by Elvidge and Lever[4] to pyridine manganese (IV)-phthalocyanine oxide, since half a molecule of oxygen is absorbed per molecule of manganese phthalocyanine. However, we have succeeded in isolating this compound as purple rhombic crystals which give a blue solution in chloroform and pyridine. Their ultraviolet absorption spectrum in pyridine corresponds with Elvidge and Lever's solution of this compound (λ_{max}, 6200 Å). The crystals are decomposed at 400° at 0.5 cm Hg to give manganese phthalocyanine. We are at present characterizing this compound still further to confirm its constitution. The third chelate prepared has been bis-(3:3':4:4':5:5'-hexamethyldipyrromethene)-manganese (II) (Fig. C). This was prepared by treating hexamethyldipyrromethene in methanol with an ammoniacal solution of manganous chloride. It crystallized from chloroform-methanol as green needles, dissolving in these solvents to give a brown solution.

These three compounds are at present being studied by ESR. Manganese phthalocyanine has a planar structure and the manganese would be expected to be a dsp^2 hybrid. The pyridine manganese (IV) phthalocyanine oxide is six-coordinate, and thus would be a d^2sp^3 hybrid. Also the bis-hexamethyldipyrromethene manganese (II) compound is tetrahedral, and thus is a sp^3 hybrid. So we have three different states of manganese and we hope to be able to recognize each and estimate it from the ESR. Preliminary experiments, at room temperature and 100°K, on crystals and solutions of these compounds have not yet been very rewarding. All have given a broad resonance over about 3000 gauss with no fine structure. However, further work is still required on these compounds before we can hope to apply it to chlorophyll preparations.

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[3] Barret, Dent, and Linstead, J. Chem. Soc. 1936, 1719.

[4] J. A. Elvidge and A. B. P. Lever, Proc. Chem. Jun/July 1959, 195.

HYDROGEN BONDING STUDIES BY NMR METHODS

Irwin D. Kuntz, Jr.

Hydrogen bonding is studied in nuclear magnetic resonance (NMR) work by noting the "chemical shift" between the signal of the bonding proton in a pure compound and the same signal when the compound has been diluted into a bonding environment. The magnitude of the shift is assumed to indicate the strength of the bond.[1] It has been found that a "downfield" shift (a shift to lower magnetic field strength at a given microwave frequency) of the proton absorption accompanies hydrogen-bond formation. Conversely, an "upfield" shift (a shift to higher magnetic field strength at a given microwave frequency) accompanies hydrogen-bond disruption. One complication in this work is the self-association of all proton donors. This has been found to range from about 0.25 part per million (shift of the magnetic field strength at which the proton absorption occurs) for $CHCl_3$ to 4 parts per million for C_2H_5OH or CH_3OH. On our instrument these values correspond to shifts in the microwave absorption frequencies of 15 and 240 cps (cycles per second) respectively.

In our work, we assumed that the behavior of a donor in a bonding medium is the sum of two effects: the disruption of self-association, and the subsequent bond formation with the environment. The first effect is observed by diluting the donor into an "inert" (nonbonding) solvent, such as CCl_4 or cyclohexane, and extrapolating the data to infinite dilution or to some standard concentration. The second effect is measured simply by observing the position of the proton resonance when the donor is added to an acceptor. By adding together the values for the two effects (according to the convention that a downfall shift in magnetic field strength is negative) one arrives at a value for the H bond formed. Whether the above assumption is correct remains to be demonstrated. However, previous authors have stated that the assumption appears to be valid.[1,2]

Experimental

The instrument used for this work was a Varian Model V-4300B High-Resolution NMR spectrometer. There was no temperature control except for a room air conditioner that was allowed to operate for some hours before a series of NMR measurements. Divergent results attributable to temperature changes were never more than 2% apart.

Accurate measurements of the "chemical shift" were facilitated by use of the side-band technique.[3] Although this procedure is capable of high accuracy (± 1%), some difficulty was experienced when the peaks involved were less than 100 cps apart. However, errors in measurement are probably not greater than 5%.

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[1] G. J. Korinek and W. G. Schneider, Can. J. Chem. 35, 1157 (1957).

[2] C. M. Huggins and D. R. Carpenter, J. Phys. Chem. 63, 238 (1959).

[3] J. T. Arnold and M. E. Packard, J. Chem. Phys. 19, 1608 (1951).

Usually seven or eight different concentrations were used in each experiment. They covered a range of 100% to 4%; a wider concentration range was not possible with the equipment used (a 5-mm probe with spinning attachment). Results (except in the case of CCl_4) were not extrapolated to infinite dilution. Instead, they are reported at 0.1 mole fraction. This approximation prevented some distortion of data and, in addition, provided comparisons with previous work on hydrogen bonding done with infrared spectroscopy.

Results

The chemical shifts observed are reported in Table XI. The values presented were determined by subtracting the chemical shift found in a 0.1 mole fraction of the donor from the shift observed with the pure compound. The CCl_4 data were extrapolated to infinite dilution; however, because of the shape of the curves, this could not be done for the other acceptors.

Correcting for self-association gives the values of relative hydrogen-bond strength. These values are reported in Table XII.

Table XI

NMR chemical shift values in hydrogen bonding

(parts per million)

Donor (0.1 molar solutions)	Acceptor				
	CCl_4	CCl_4, inf. dil	Et_3N	Et_2O	C_3H_7I
MeOh	1.16		0.82	2.05	2.20
	1.25[a]	4.28[a]			
Et_2NH	0.31	0.37	-0.73	-0.45	---
EtSH[b]	0.12-0.15	0.17	0.12	0.22	---

[a]Data of M. Saunders and J.B. Hyne, J. Chem. Phys. 29, 1319 (1958).

[b]Data approximate only.

Table XII

Relative hydrogen-bond strengths in terms of NMR chemical shifts (parts per million)

Donors		Acceptors			
Compound	Self-Association Value	Et_3N	Et_2O	C_3H_7I	C_3H_7F
MeOH	1.16	0.34	-0.89	-1.04	--
Et_2NH	0.31	1.04	0.76	--	--
EtSH	0.15	0.03	-0.07	--	--
$CHCl_3$ [a]	0.30	1.15	0.70	--	--
$SiHCl_3$ [b]	0.04	--	--	--	0.30

[a] Data of Korinek and Schneider[1]

[b] Data of Huggins and Carpenter[2]

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