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Ernest O. Lawrence Radiation Laboratory

BIO-ORGANIC CHEMISTRY QUARTERLY REPORT
March, April, May 1960

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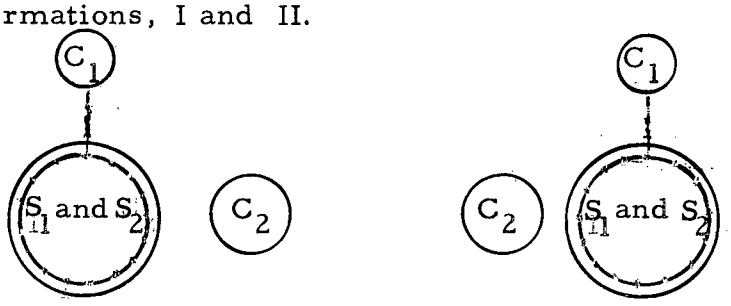
THE ENERGY BARRIER BETWEEN THE ENANTIOMERS OF 1, 2-DITHIANE

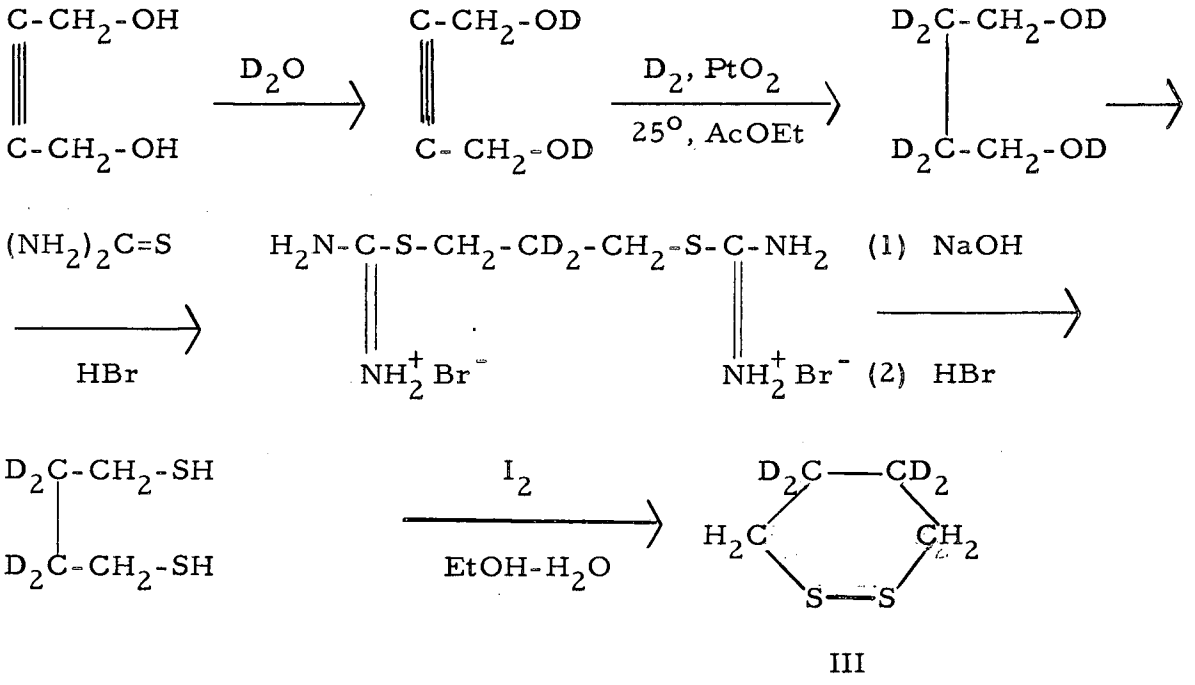
Goran Claeson and Gaylord Androes

The dihedral angle in an organic disulfide can produce two different molecular conformations, I and II.



I II

These are optical antipodes, and their stability depends on the height of the rotational barrier hindering free rotation about the disulfide bond. We have studied the intercoversion between the enantiomers of the cyclic disulfide 1, 2-dithiane by nuclear magnetic resonance. This molecule gives, however, a very complicated NMR-spectrum (Fig. 1), and therefore we prepared a deuterium-substituted derivative (III) as follows:



III

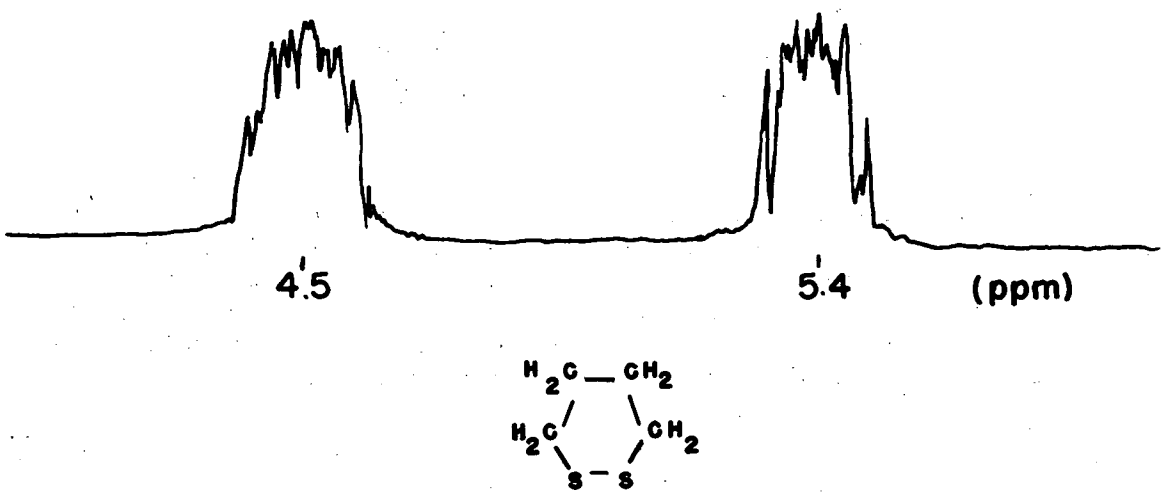


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Fig. 1. Proton resonance spectrum at 60 Mc of 1, 2-dithiane at room temperature referred to benzene (50% solution in benzene).

From low-temperature studies of a 1M solution in carbon disulfide we obtained data that provide an estimate of the rotational barrier for this molecule.

At temperatures below -50° the spectrum consists of a quartet of the AB type[1] (Fig. 2). The maximum separation of the two inner peaks (2C-J) is 10.4 counts/sec. The coupling constant (J), equal to the separation of either the right-hand pair or the left-hand pair,[1] is 13.0 counts/sec. This shows that the H-C-H bond angle is normal.[2] When the temperature is raised above -50°, the peaks in the quartet start to converge, and at -43° the spectrum consists of one single broad peak. As the temperature increases above -43° the peak sharpens and remains constant up to the highest temperature studied (150°).

From the low-temperature pattern, the chemical shift between the axial and equatorial hydrogens, $\nu_a - \nu_e$, was calculated as 18.5 counts/sec ($\nu_a - \nu_e = \sqrt{4C^2 - J^2}$). We assumed that the following relation for the rate of interconversion[3] holds at -43°, the temperature at which the peaks coalesce:

$$k = \frac{\pi(\nu_a - \nu_e)}{\sqrt{2}} .$$

From this we calculated the rate constant $k = 41.1\ \mathrm{sec}^{-1}$. An exact value for k in this case may be calculated from quantum mechanical expressions given by Kaplan,[4] but such a calculation would probably not change k significantly.

---

[1] J. A. Pople, W. G. Schneider, and H. J. Beinstein, High Resolution Nuclear Magnetic Resonance (McGraw-Hill Book Co., New York, N. Y., 1959), p. 119.

[2] M. Karplus, D. H. Anderson, T. C. Farrar, and H. S. Gutowsky, J. Chem. Phys. 27, 597 (1957);
M. Karplus and H. S. Gutowsky, Abstracts, Am. Chem. Soc. Meeting, San Francisco, 1958.

[3] H. S. Gutowsky and C. H. Holm, J. Chem. Phys. 25, 1228 (1956).

[4] J. Kaplan, J. Chem. Phys. 28, 278 (1958).

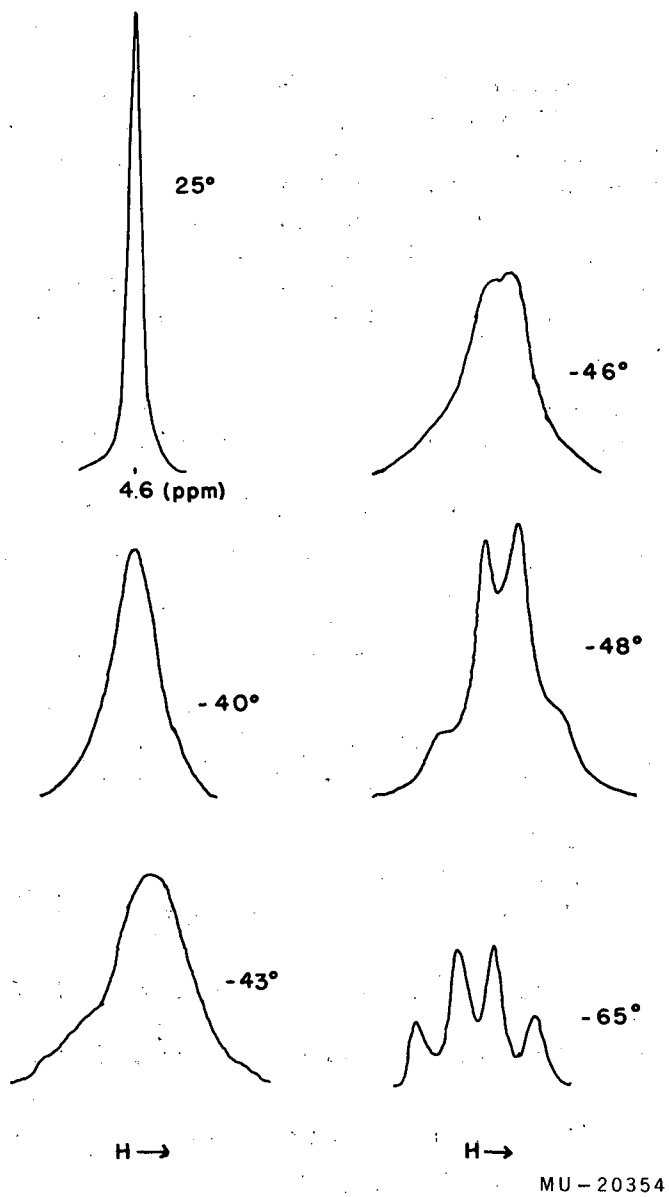
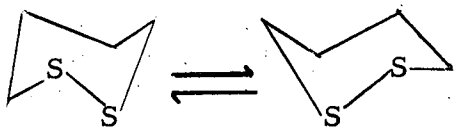


Fig. 2. Proton resonance spectra at 60 Mc of 1, 2-dithiane-4, 5-D_4 in CS_2 at different temperatures. Benzene as internal standard.

If the interchange from one isomer to the other



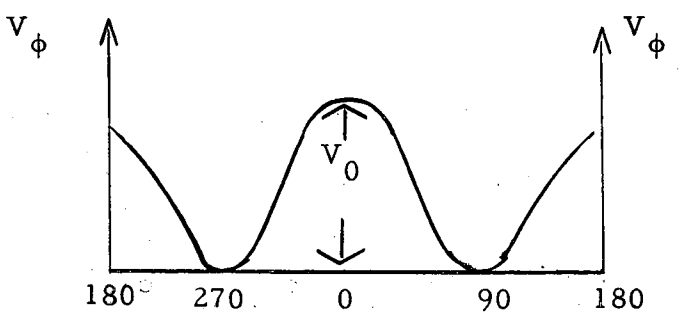
is treated as a typical rate process, and the transmission coefficient (c) is equal to unity, then Eyring's equation gives $\Delta F^* = 11.6$ kcal/mole. Thus, the

$$k = c \cdot \frac{RT}{Nh} \cdot \exp(-\Delta F^*/RT)$$

molecular conformations have a very short lifetime. Even at -43° the half-life for the enantiomers is only 0.02 sec. The same measurements on cyclohexane[5] with similar assumptions gives a value of 9.7 kcal for ΔF^*. Noting that only three methylene oppositions are involved for the transition in dithiane as compared to six for cyclohexane, one estimation of the ΔF^* for disulfide rotation alone (in the cyclic compound) might be about 6.8 kcal/mole (11.6 kcal/mole - $\frac{9.7}{2}$ kcal/mole = 6.8 kcal/mole). Since the maximum dihedral angle in the six ring is likely to be about 60 deg, the energy difference (ΔF^*) between 0 deg and 90 deg would be 9.1 kcal/mole, assuming the following angular dependence:[6]

$$V_\phi = 1/2 V_0 \left[1 - \cos 2(90-\phi) \right],$$

ϕ = dihedral angle.



Dihedral angle, ϕ (deg)

[5] F. R. Jensen, D. S. Noyce, C. H. Sederholm, and A. J. Berlin, J. Am. Chem. Soc. 82, 1256, (1960).

[6] M. S. Newman, Steric Effects in Organic Chemistry (John Wiley and Sons, Inc., New York, N. Y., 1956), p. 55.

The entropy of activation (ΔS^*) for this reaction is not known, however, and we must await a direct measurement of the temperature coefficient of k to evaluate the heat term (ΔH).[7] Since the entire transition in the spectrum from a single line to a quadruplet occurs between -40° and -50°, precise temperature control and simpler spectra would be desirable. Experiments toward this end are in progress.

By comparing the infrared spectra (Fig. 3) of 1, 2-dithiane and its deuterium-substituted derivative (III), assignments of the C-H stretching vibrations in the 3000- to 2800-cm^{-1} region were possible. The normal C-H vibrations[8] for CH_2 are 2926 and 2853 ± 10 cm^{-1}. The α CH_2 peaks are shifted to 2951 and 2913 cm^{-1} as determined from the deuterium compound. The two remaining peaks, of the four seen in the 1, 2-dithiane spectrum, must be assigned to β CH_2 and are located at 2925 and 2846 cm^{-1}. These peaks are nearly at their normal positions, and are thus very little affected by the sulfur atoms. Pozefsky and Goggeshall have tried to correlate the C-H stretching in compounds containing sulfur, but they had difficulties in assigning the different bands to respective groups.[9]

Experimental

1, 4-Butynediol-D_2

2-Butyne-1, 4-diol (Aldrich Chemical Co.) was recrystallized from ethyl acetate, m. p. 54-57°. 64.5 g (0.75 mole) of this diol was dissolved in 60 ml of D_2O (99.5%). The water was distilled off at reduced pressure. Benzene was added and distilled off to remove the last trace of water. The same procedure (dissolving in 60 ml of D_2O and removing the water) was repeated twice.

1, 4-Butanediol-D_2-2, 3-D_4

The resultant diol was dissolved in 150 ml of hot ethyl acetate. The solution was cooled and approximately 0.2 g PtO_2 was added. The solution was deuterated by shaking in a 450-ml bottle under a deuterium gas pressure of about 30 lb/in.^{2} After 15 hours, the calculated amount of gas had been consumed. The PtO_2 was filtered off and the solvent distilled at reduced pressure. Fractionation gave 52.5 g of a colorless liquid; bp, 121-123°/10 mm; n_D^{21} = 1.449; yield 73%.

1, 4-Butanedithiol-2, 3-D_4

In a 500-ml three-necked round-bottom flask, fitted with a stirrer and reflux condenser, were placed 36.5 g of 1, 4-butanediol-D_2-2, 3-D_4

[7] D. W. Scott, H. L. Finke, M. E. Gross, G. R. Guthrie, and H. M. Huffman, J. Am. Chem. Soc. 72, 2424 (1950).

D. W. Scott, H. L. Finke, J. P. McCullough, M. E. Gross, R. E. Pennington, and G. I. Waddington, J. Am. Chem. Soc. 74, 2478 (1952);

G. Bergson and L. Schotte, Arkiv Kemi 13, 43 (1958).

[8] L. J. Bellamy, The Infrared Spectra of Complex Molecules (Methuen and Co. Ltd, London, 1958), p. 13

[9] A. Pozefsky and N. Goggeshall, Anal. Chem. 23, 1611 (1951).

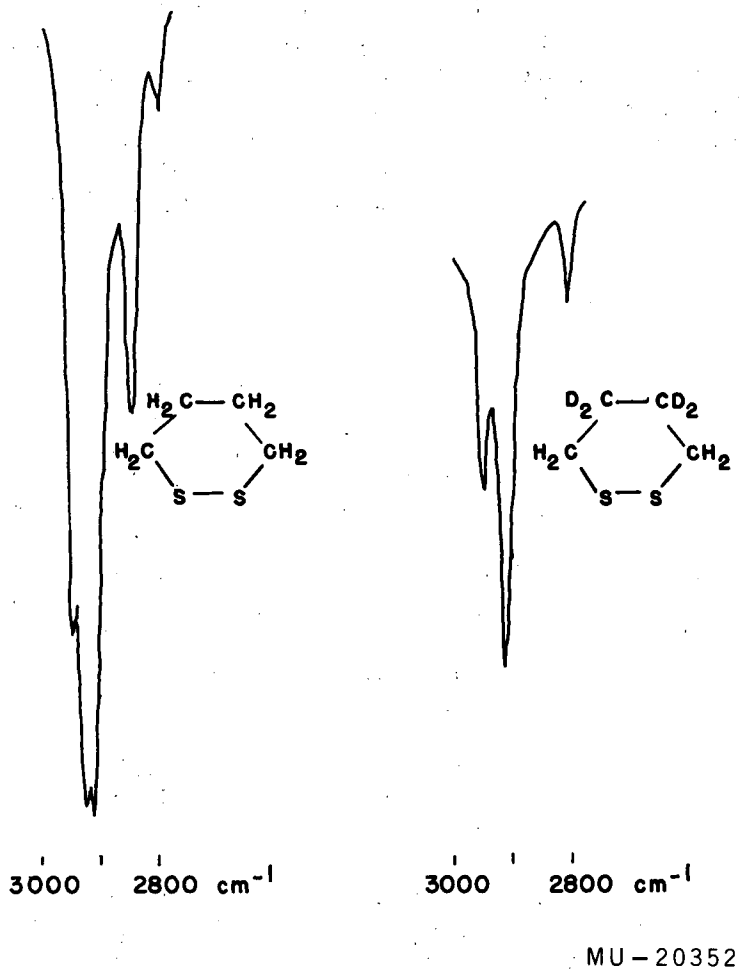


Fig. 3. Infrared absorption spectra (300 to 2800 cm^{-1}) of 1, 2-dithiane and 1, 2-dithiane-4, 5-D_4.

(0.38 mole), 60.8 g of thiourea (0.8 mole), and 280 ml of 47% hydrobromic acid (2.4 mole). The mixture was refluxed for 8 hours with stirring. The flask was allowed to cool (the thiouronium salt was then precipitated as a fine crystalline solid), and a solution of 96 g (2.4 mole) of sodium hydroxide in 200 ml of water was added. The mixture was refluxed for 2 hours while a stream of nitrogen was bubbled through the solution. The solution was cooled, and the two phases were separated. The acidified aqueous layer was extracted with three 100-ml portions of ether. The combined organic layers were dried over calcium sulfate. The ether was removed at reduced pressure and the remaining oil fractionally distilled. bp, 79-81°/17 mm; yield, 21.6 g (45%). (A fore-run of 5 g boiling at 31°/17 mm could be tetrahydrothiophene, bp 119°.)

1, 2-Dithiane-4, 5-D_4

21.6 g (0.171 mole) of 1, 4-butanediol-2, 3-D_4 was dissolved in 2 liters of ethanol-water. A 5% alcoholic-iodine solution was added with stirring until a faint yellow color persisted. A few drops of 1% sodium thiosulfate solution was added to make the solution colorless. Water was added to a total volume of 5 liters and the solution extracted with two 150-ml portions of petroleum ether. After the solution was dried over calcium sulfate, the solvent was removed by distillation. The remaining disulfide was distilled at 34 mm, and solidified in the condenser: bp, 87-88°/34 mm; yield, 18 g (85%). The compound was easily sublimed to give crystals with mp 32-33°. The NMR spectrum of the sublimed compound showed that it was about 99% isotopically pure.

The infrared spectra were obtained with a Beckman infrared IR-7 spectrophotometer. The substances were examined as solids in pressed potassium bromide plates.

A Varian high-resolution NMR spectrometer V-4311, operating at 60 Mc, was used to obtain the NMR spectra. The signal displacements in the spectra were measured with the side-band technique.

FURTHER INVESTIGATION OF ORGANIC MATTER IN METEORITE MURRAY

Susan V. Himes

The investigation of meteorite Murray has been reported in two previous Quarterly Reports.[1, 2] This study has now been narrowed to examination, by various methods, of the water extract of the meteorite. Table I gives an elemental analysis of the water extract. The 5% of sulfur was bound in such a way (i.e., organically) that it could be combusted. The 10% of sulfur was determined from the residue, but calculated from the total weight of the extract, and was probably in the form of sulfate.

Table I

Elemental analysis of water extract of Meteorite Murray

Element	Percent
C	5.98
H	2.83
N	0.55
S combustible	5.12
S from residue	10.34
P	0.0
Residue	62.4

Making the gross assumption that the 62% residue constitutes all the inorganic material in the water extract and that the other 38% constitutes water and the organic material, one can recalculate the elemental analysis on

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

[2] Susan K. Vaughn and Melvin Calvin, in Bio-Organic Chemistry Quarterly Report, UCRL-9041, Dec. 1959, p. 24.

an inorganic-free basis

Element	Percent
C	15.7
H	7.5
N	1.5
S	13.5

This still gives quite a low value for carbon.

However, since the sulfur is water-soluble organic sulfur, one plausible form for it is organic sulfate. Assuming that all the sulfur is in this form, one calculates that there is 27% oxygen associated with it. Another elemental analysis can be made on an organic-sulfate-free basis:

Element	Percent
C	26.2
H	12.5
N	2.4

It is a reasonable assumption that the remaining 59% is oxygen. Since there undoubtedly was a significant but unknown amount of water present in the extract, we shall further assume that 2.5% of the hydrogen is in the form of water. Then there would be 20% oxygen associated with it, and removing the corresponding value for water from the analytical data, one has left 26.2% carbon, 10% hydrogen, 2.4% nitrogen, and 39% oxygen. The water-, organic-sulfate-, and inorganic-free analysis is:

Element	Percent
C	33.8
H	12.9
N	3.1
O	50.0

This now approaches the value for carbon in organic substances such as pyrimidines and sugars. (Both have approximately 40% carbon.) The formula for uridine, a pyrimidine attached to a sugar, is $C_4H_3N_2O_2$-$C_5H_9O_4$. Its elemental analysis is:

Element	Percent
C	44.2
H	4.9
N	39.2
O	11.5

The biggest discrepancy between the preceding analysis and this one is the very low value for nitrogen. To accommodate the suggestion of the presence of sugars, it was reported earlier that a silver nitrate test performed on a sample of unfractionated water extract did indicate the presence of reducing compounds.[2]

An x-ray-diffraction pattern of a portion of the water extract was obtained in order to determine the identity of the inorganic salt.[3] It was shown that the water extract contains mostly $CaSO_4 \cdot 2H_2O$.

The first technique for investigating the water extract was fractionation on a Dowex-1-Cl anion-exchange column. One such experiment has already been reported.[2] To verify those results, another water extract of Meteorite Murray was submitted to a similar fractionation process. The procedure was as follows: A sample of water-extracted material, usually about 200 mg, was dissolved in a minimum amount of water (about 50 to 60 ml) and then applied to a Dowex-1 column in the chloride form (0.9 cm diameter, 12 cm length; holdup volume approximately 35 ml). The column was washed through with water until the eluate was free from weighable material and until no ultraviolet absorption appeared in the eluate. (A Gilson Medical Electronics fraction collector was used; it automatically recorded the ultraviolet absorption of the eluate at 259 mμ. Ten-ml fractions were collected.) Then the column was eluted with 0.05 N HCl until neither ultraviolet absorption nor weighable material remained. Each 10-ml fraction that had ultraviolet absorption or weighable material was evaporated to dryness, the material weighed, and the ultraviolet absorption spectrum taken in aqueous solution.

It was definitely determined that the ultraviolet-absorbing material that comes off the column was not contamination from the column. Before a meteorite sample was placed on the column, the column underwent the same procedure of water washing, followed by HCl elution. Random 10-ml fractions were evaporated to dryness and each contained less than 1 mg of weighable material. The ultraviolet absorption was insignificant. Fifteen fractions were mixed together and evaporated to dryness. Again, the amount of weighable material and the uv absorption were both insignificant. This blank determination was made before every application of sample to the column.

---

[3] We are grateful to Mrs. Helena Ruben for the x-ray-difraction pattern.

Since the water extract is mainly $CaSO_4$, it was suggested that perhaps the calcium sulfate was displacing something from the column. To test this possibility, 200 mg of $CaSO_4$ dissolved in a minimum amount of water (approximately 70 ml) was applied to a Dowex-1-Cl column (0.9 cm diameter, 15 cm length), washed with water, and then eluted with 0.05 N HCl. It was previously determined that the $CaSO_4$ had no ultraviolet absorption, and, since $CaCl_2$ would be washed off the column, it was shown that $CaCl_2$ had no uv absorption. The $CaCl_2$ came through the column in about the first ten fractions of the water wash. None of these had significant ultraviolet absorption. Thereafter, through the rest of the water wash followed by the HCl elution, no significant amount of weighable material or ultraviolet-absorption was detected. Therefore, the $CaSO_4$ does not displace ultraviolet-absorbing material from the column.

A 255-mg portion of the water extract of Murray was applied to a Dowex-1-Cl column and eluted as described above. The ultraviolet absorption of the sequence of fractions is shown in Figs. 4 and 5. Figure 4 is a plot of the fractions against the percent transmission that is recorded automatically as the eluate flows past the ultraviolet lamp of the fraction collector. Figure 5 is a plot of the fractions against their ultraviolet absorption at 270 mμ after they were evaporated to dryness, put into solution again, and examined with a Cary Spectrophotometer. Table II shows the amount of material in each fraction. Thus, in agreement with the earlier experiment,[2] it appears that the HCl elutes some material with high ultraviolet absorption. The complete absorption spectra of a few of these fractions are shown in Fig. 6.

In order to verify the results previously reported,[2] the effect of pH on the spectra of two of these fractions was determined. The pH sequence spectra were obtained with a 1-ml portion of a water-extract fraction diluted to give an optical density at 270 mμ of approximately 1.2. The 1.0 ml was put into a Pyrocell "Supersil" semimicro cell with a 1-cm path length, and 1.0 ml of water was placed in an identical cell as reference. The pH was changed by adding to each cell from a microburet an appropriate amount, usually 1 to 5 λ, of 5 N HCl or NaOH.[4] The approximate pH was determined with pH paper. Figure 7 shows the effect of pH on Fraction 1 of the HCl elution. The sharp peak at 272 mμ is decreased and shifted to 268 mμ on going from pH 4 to pH 8. This result is similar to the one previously reported[2] and indicates a pK value between 4 and 8. Figure 8 shows the effect of pH on Fraction 10 of the water wash. Here, also, a reversible change is noticed. However, it is quite different and not as well defined as that of the HCl fraction. Here a pK value seems to be present between 5 and 2.

An infrared spectrum of a portion of the HCl Fraction 2 was obtained after it was dried and smeared on a NaCl plate (Fig. 9). This seems to be no more helpful than the infrared spectra already reported.[2] There is some type of carbonyl in the 1700 cm^{-1} region, weak CH absorption at 2900 cm^{-1}, and other absorption, ill defined, between 1400 and 1100 cm^{-1}.

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[4] Appropriate blanks were run to make sure that the NaOH and HCl had no ultraviolet absorption.

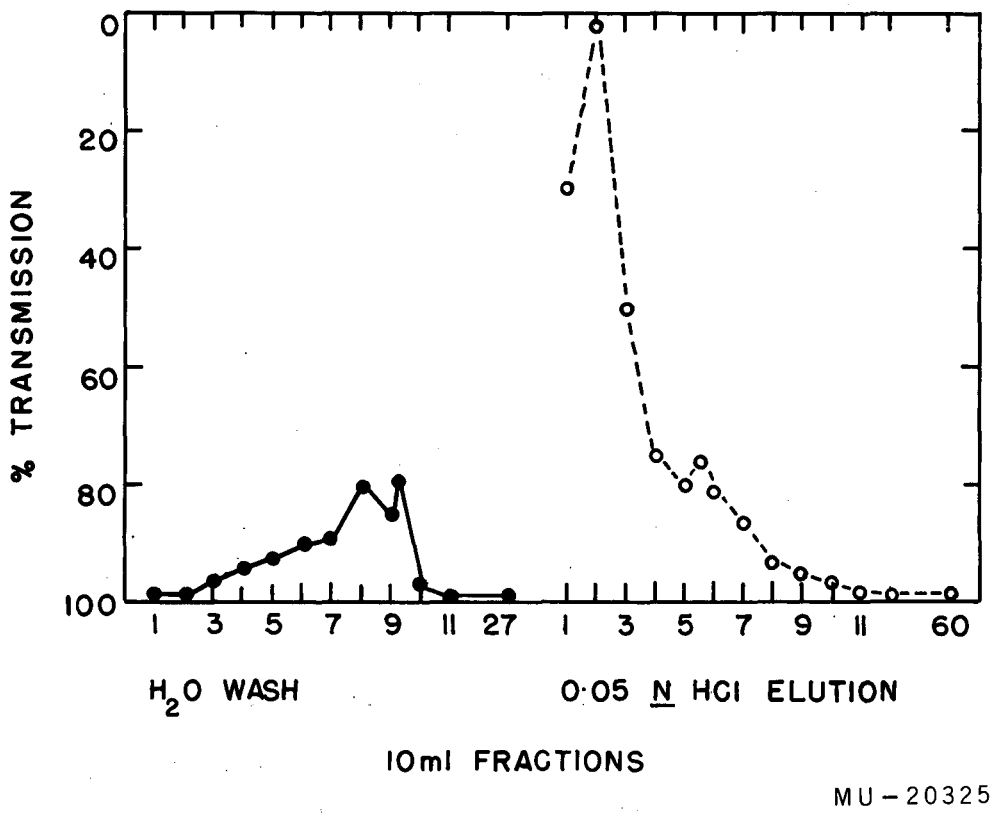

Fig. 4. Water extract of Murray (Expt. D). Automatically recorded uv absorption (259 mμ) of fractions off Dowex-1-Cl.

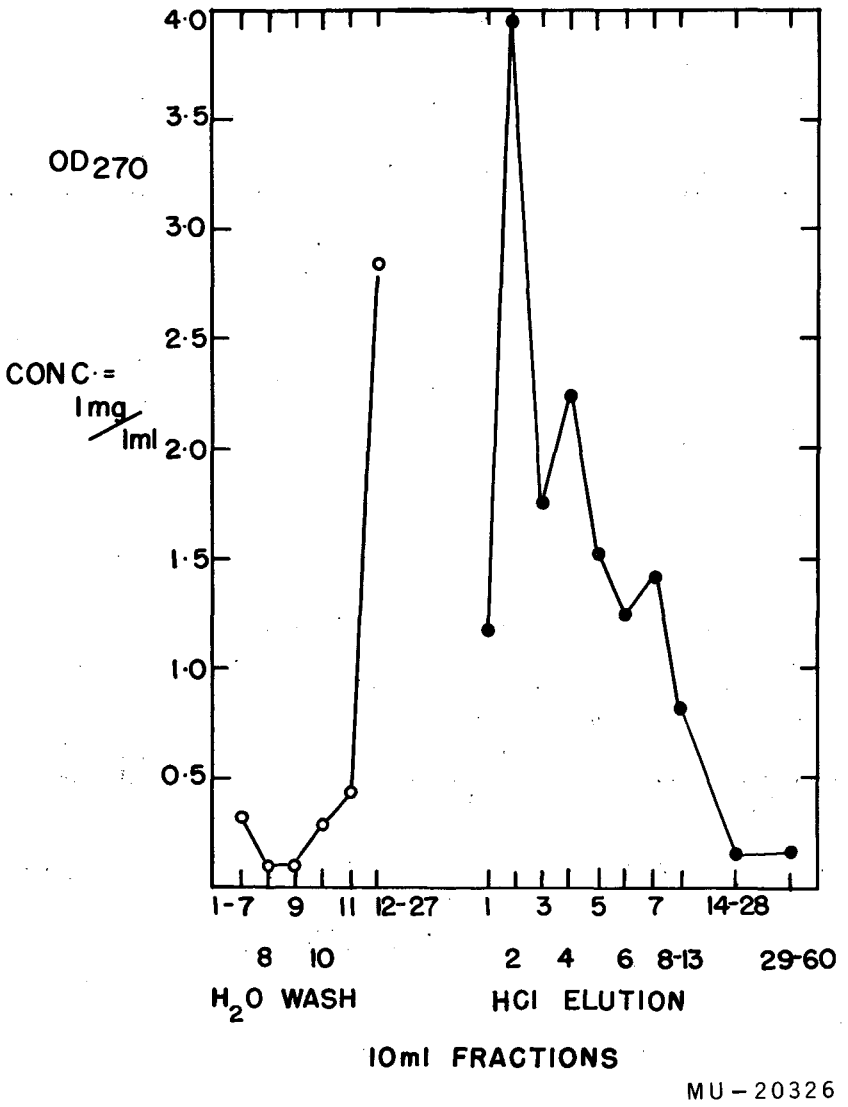

Fig. 5. Water extract of Murray (Expt. D). Specific uv absorption (270 mμ) resulting from fractionation on Dowex-1-Cl.

Table II

Water extract of Meteorite Murray (Expt. D).
Weight of material in each fraction off Dowex-1-Cl column

Treatment	Fraction	Weight (mg)
H_2O wash	1-7	168
	8	37
	9	35
	10	12
	11	2.5
	12-27	2.9
0.05 N HCl elution	1	2.6
	2	5
	3	3.5
	4	2.2
	5	3.6
	6	1.4
	7	1.7
	8 - 13	5.3

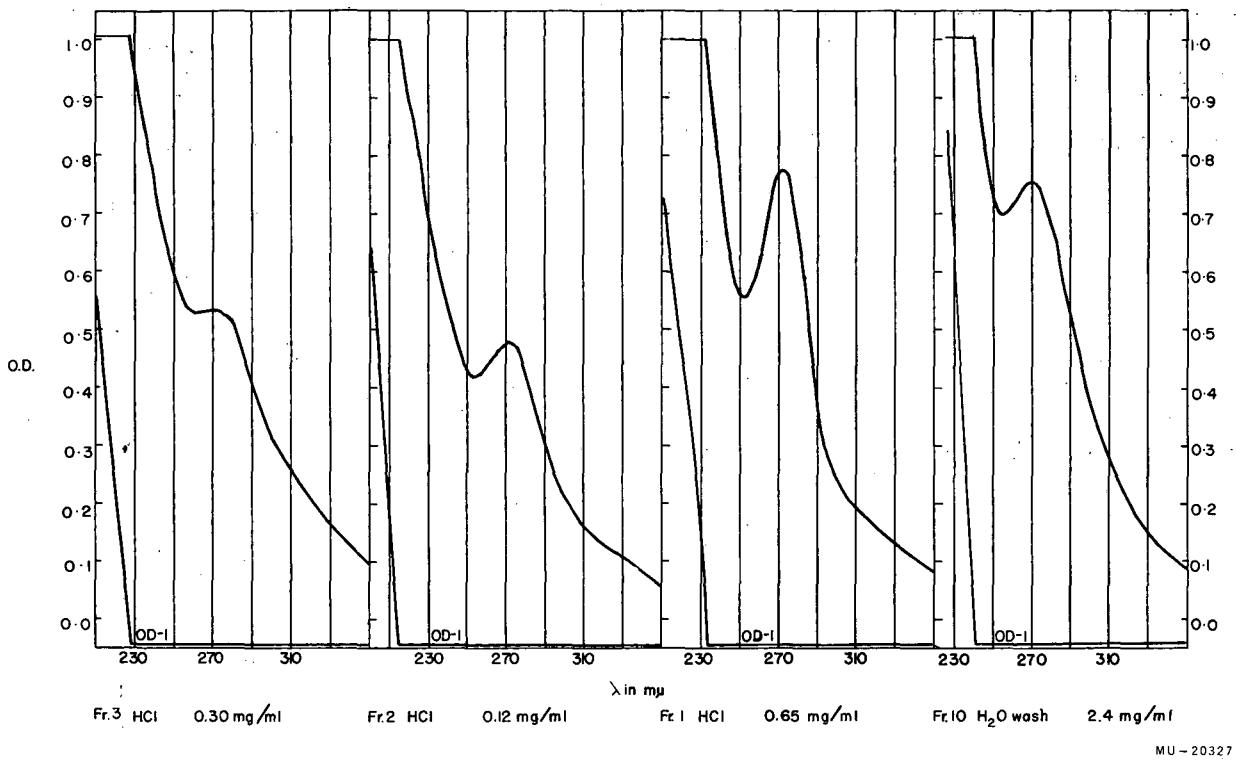

Fig. 6. Water extract of Meteorite Murray (Expt. D). Complete uv spectra of fractions from Dowex-1-Cl.

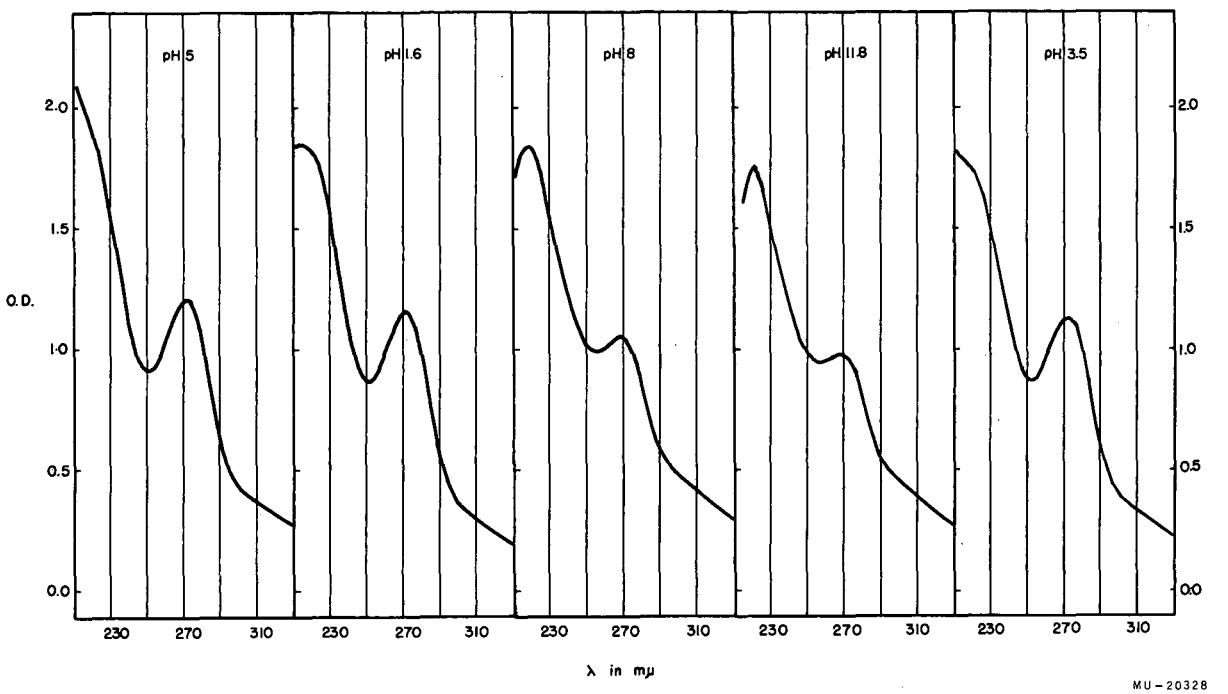

Fig. 7. Water extract of Meteorite Murray (Expt. D). Effect of pH on Fraction 1-HCl.

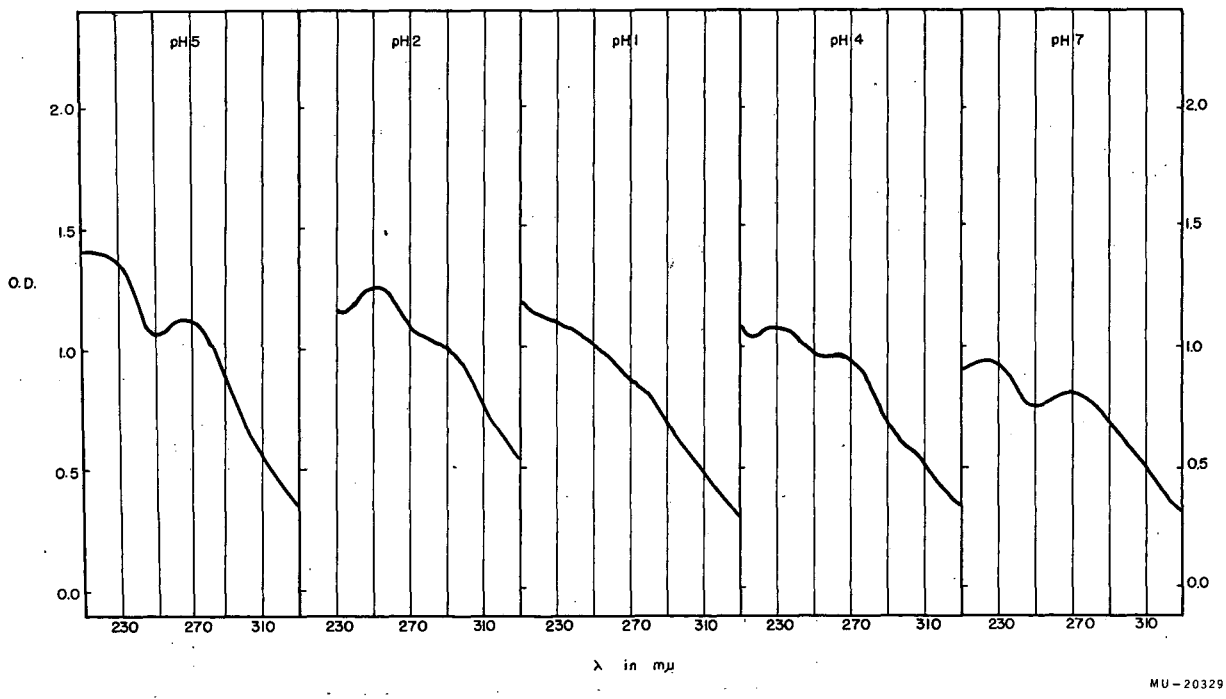

Fig. 8. Water extract of Meteorite Murray (Expt. D). Effect of pH on Fraction 10, water wash.

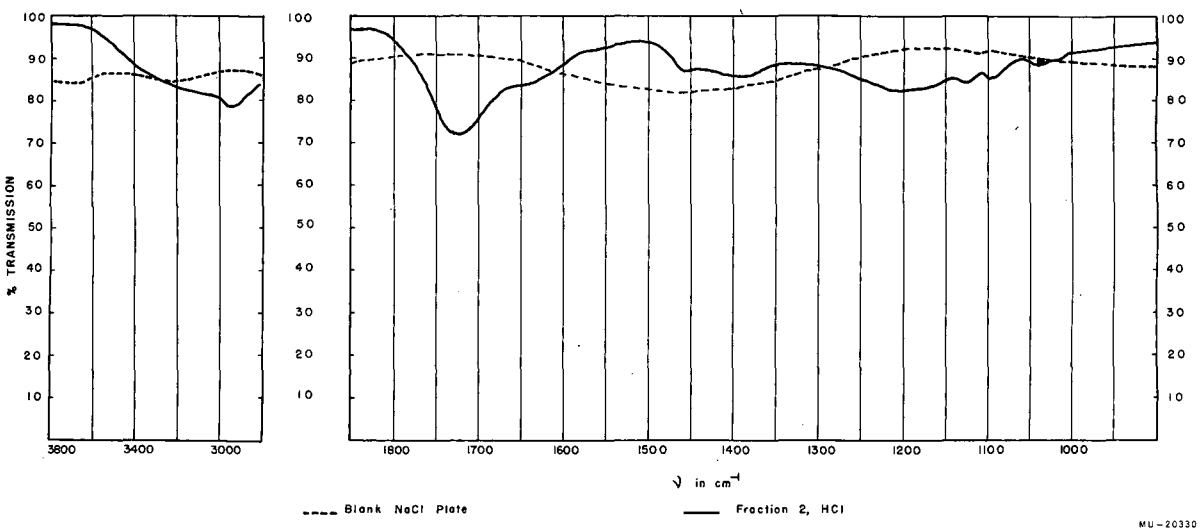

Fig. 9. Water extract of Meteorite Murray (Expt. D). Infrared spectrum of Fraction 2-HCl, in solid state.

The fluorescence of several of the water-extract fractions was investigated by using a fluorescence attachment on the Cary Model 14 Spectrophotometer. Two filters for producing activating light are provided with the instrument. A 1.0-cm path of $NiSO_4$ solution (500 g/l) and a specially ground Corning No. 9863 Corex filter provide light at 2537 A. However, the light is not monochromatic, but extends from 2537 to 3700 A. A Corning No. 5840 filter provides nearly monochromatic light at 3658 A.

First, blanks were run (water in the 1-cm Pyrocell Supercil cell and a clean Pyrex plate) to make certain that the holders did not fluoresce. Then the fluorescences of two known single compounds--fluorescein in solution and solid anthracene on the Pyrex plate--were observed in order to ascertain that the instrument was operating correctly. Figures 10 and 11 show the fluorescence of some of the water extract samples with the 254- and 366-mμ filters respectively. In the figures, the width of the fluorescence band is indicated and the peak is roughly sketched. All the bands end abruptly at a certain wave length on the short-wave-length side because there the photocell is strongly affected by scattering of the activating light. As a result, the recorder pen goes off scale. Fraction 2 of the HCl elution was observed both as a smear on the Pyrex plate and in solution. In the latter state, its spectra are the same as those of the other fractions in solution. With the 254-mμ filter, Fraction 2, as a solid, has the strong narrow band at 545 mμ that is observed as a shoulder in the other spectra. One observes that with the 254-mμ filter the fluorescence bands of the fractions in solution peak near 400 mμ; with the 366-mμ filter, they peak near 430 mμ.

It is interesting to note that the water-wash sample, which includes all but the first seven water-wash fractions, was 10 times as concentrated as the HCl-elution fractions, yet its fluorescence intensity was the same or slightly lower. Therefore, the fluorescent material was mainly held up on the column until the HCl elution. Fraction 14-28-HCl was also ten times as concentrated, yet its fluorescence intensity was somewhat lower. Therefore, the fluorescence could not be due to something in the HCl, since there was 15 times as much HCl in this fraction as in the single fractions. Of course, in order to use concentrations as high as 1 mg/ml and more, it is assumed that the amount of fluorescent material is quite small. If this is not true, then self-quenching may be occurring and the results may be spurious.

The only clues as to what might be producing this fluorescence come from the literature. Duggan et al. report that there are two types of organic compounds that produce fluorescence: conjugated unsaturated types, and aromatics with an electron-donating group.[5] This would include phenols, aromatic amines, indoles, polycyclic aromatics (e.g., purines), and conjugated polyenes. Pyrimidines have no measurable fluorescence. Bowen and Wokes report that very few inorganic compounds fluoresce in solution, and most organic substances have broad, structureless bands.[6] Inorganic salts give narrow bands which are almost line spectra.[7]

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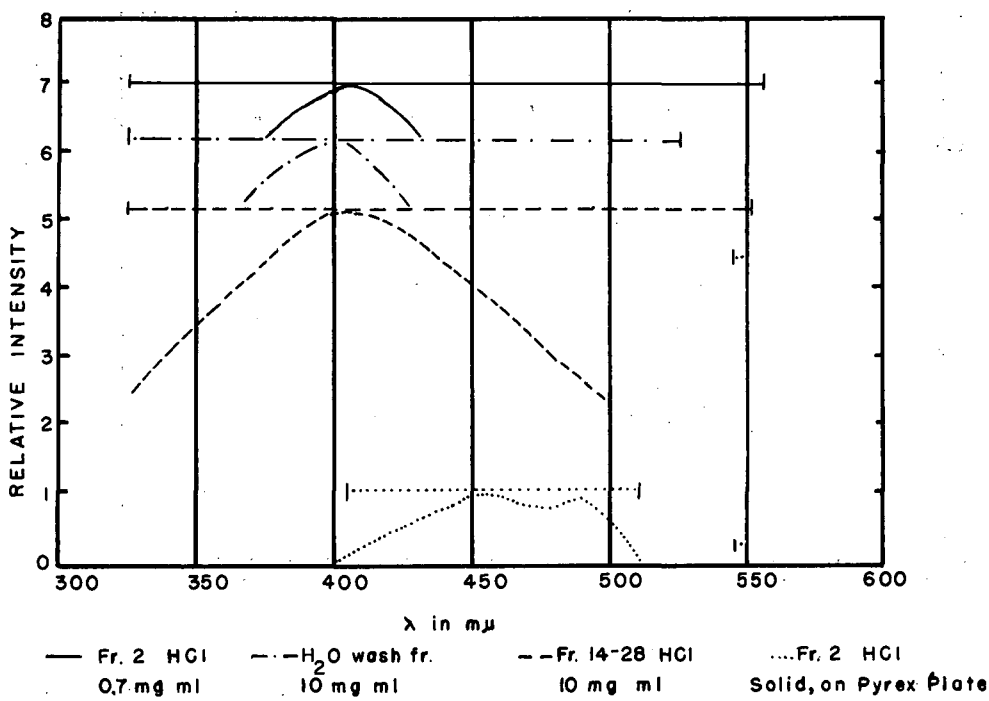
[5] Duggan, Bowman, Brodies, and Udenfriend, Arch. Biochem. Biophys. 68, 1 (1957).

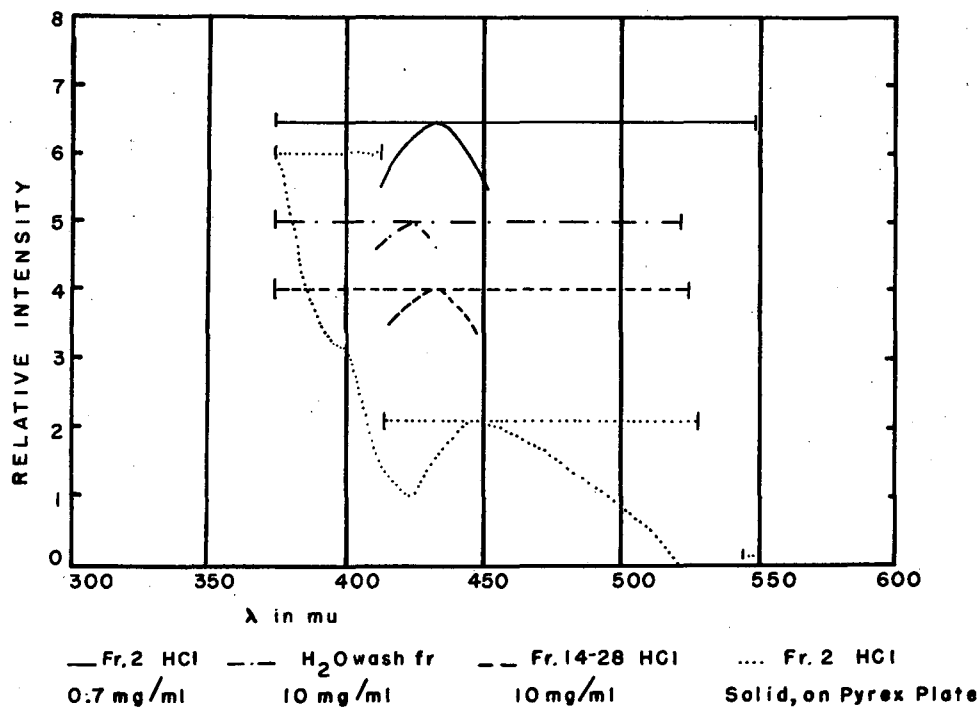
[6] Bowen and Wokes, Fluorescence of Solutions (Longmans, Green and Co., London, 1953).

[7] C. E. White in Trace Analysis, Yoe and Koch, eds. (John Wiley and Sons, New York, 1957), p. 211.



MU-20323

Fig. 10. Water extract of Meteorite Murray (Expt. D). Fluorescence of fractions from Dowex-1-Cl, 254-mμ filter. The broad band in the spectrum of two of the samples in solution had a shoulder of low intensity at 545 mμ. As shown in the figure, Fraction 2-HCl as a solid had a narrow band of high intensity at that wave length, and the water-wash fraction had weak fluorescence there.



MU-20324

Fig. 11. Water extract of Meteorite Murray (Expt. D). Fluorescence of fractions off Dowex-1-Cl, 366-mμ filter. As shown in the figure, Fraction 2-HCl as a solid had weak fluorescence at 545 mμ in addition to its broad band.

Paper chromatography was attempted on a portion of Fraction 2-HCl. Approximately 0.1 mg was applied onto a piece of 23-cm-square Whatman No. 1 filter paper. Observed over a uv lamp, the spot showed only purple fluorescence, with no dark absorption. The solvent used for the first dimension of chromatography was isobutyric acid/conc. NH_4OH/water in a ratio of 66:1:33.[8] This is a good solvent for separating nucleic acid bases, nucleosides, and nucleotides. After it was run for about 4 hours, the chromatogram was dried and observed over the uv lamp. Some fluorescence remained at the origin; the rest had traveled with the solvent front, with some streaking behind. Butanol/glacial acetic acid/water (4:1:1) was the solvent for the second dimension; this solvent has been used for separating pteridines.[9] Again, after it was run for about 4 hours, the chromatogram was dried and observed over the uv lamp. Still some fluorescence remained at the origin. The fluorescence observed at the solvent front in the first dimension streaked down to the solvent front in the second dimension. Since there were no definitely separated spots, the only conclusion that can be drawn is that there is present a mixture of substances. Further fractionation seems to be necessary, perhaps by means of a series of acid elutions of increasing concentrations.

The uv spectra of compounds that might possibly be related to the unknown were examined for any resemblance. The spectrum of potassium tetrathionate, $K_2S_4O_6$, purchased from Fairmount Chemical Co., Inc., Newark, N. J., showed strong but smooth end absorption beginning at 290 mμ, with no indication of peaks or of recognizable features. The spectrum of benzylamine has a sharp peak at 250 mμ and a minimum at 230 mμ. This is dissimilar, since the unknown shows a minimum at 250 mμ. Marshall and Walker report on some potentially tautomeric 2-and 4(6)-substituted pyrimidines.[10] One of these compounds with a peak near 270 mμ and a minimum near 250 mμ, 1,4,6-trimethyl-2-thiopyrimidone, was synthesized from acetylacetone and methylthiourea according to the method of Hale and Williams.[11] The extinction coefficient at 270 mμ reported by Marshall and Walker was 1.6×10^4. The synthesized compound gave $\epsilon_{270} = 1.8 \times 10^4$. When this compound was subjected to the pH-change procedure, the shape of its spectrum was slightly affected by acid and not at all by base.

It would be well to summarize what is known about the compounds in the water extract. From the paper chromatography, it appears that a mixture is present, including something that remains at the origin in two solvents. The uv absorption shows a peak near 270 mμ and minimum near 250 mμ; the absorption is affected reversibly by pH. It is held back on Dowex-1-Cl and is eluted by comparatively strong HCl. It fluoresces near 400 mμ. The fluorescence does not preclude the possibility that a pyrimidine is present, but does indicate that something else must also be present.

[9] F. Korte, in Ciba Foundation Symposium on Chemistry and Biology of Pteridines (Little Brown and Co., Boston, 1954), p. 163

[10] J. R. Marshall, and James Walker, J. Chem. Soc., 1004 (1951).

[11] Hale and Williams, J. Am. Chem. Soc., 37, 598 (1915).

THE RADIATION DECOMPOSITION OF $[(CH_3)_3NCH_2C^{14}H_2OH]^+Cl^-$

Margaret A. Smith and Richard M. Lemmon

Recent work in this Laboratory has led to proposals for the mechanism of the radiation decomposition of crystalline choline chloride.[1] These proposals assume the transient existence of a radical with a structure similar to $:CH_2 \cdot CH_2OH$, such a structure being based on the ESR spectra obtained from selectively deuterated, irradiated choline chloride. It is also quite possible that the radical might assume an ethylene oxide structure, such as $\underset{O}{\overset{CH_2 \cdot CH_2}{\diagdown\!\diagup}}$, since it is known that the unpaired electron is shared equally by the two carbon atoms. Such a symmetrical intermediate would mean that the two carbon atoms initially present in the ethanol group of choline would be indistinguishable in the acetaldehyde that appears as a result of the radiation decomposition. To test this possibility we have synthesized choline chloride labeled with C^{14} in the carbinol carbon atom and have observed the distribution of C^{14} in the two carbon atoms of the radiation-produced acetaldehyde.

Experimental Procedure

The general method for the preparation of $[(CH_3)_3NCH_2C^{14}H_2OH]^+Cl^-$ was the condensation of ethyl bromoacetate-1-C^{14} with dimethylamine to give the ethyl ester of N, N-dimethylglycine, reduction of the latter compound to N, N-dimethylaminoethanol, and quaternization with methyl iodide. This sequence of reactions is described below.

Ethyl ester of N-N-dimethylglycine-1-C^{14}

To a petroleum ether solution of 1.5 millicuries of ethyl bromoacetate-1-C^{14} was added sufficient inactive compound to give a total of 1.5 millimoles. The solution was added dropwise, with stirring, to a solution of 6 mmoles of dimethylamine in petroleum ether at 0°. The reaction mixture was stirred at that temperature for 1 hour and then allowed to come to room temperature slowly. The bulk of the excess amine, and approximately one-half the solvent, were removed by evaporation with the aid of an air stream. The remaining organic solvent was removed in a similar manner after the addition of several ml of 0.1 N hydrochloric acid. The acid solution of the product and excess dimethylamine was made basic with a saturated $NaHCO_3$ solution, and nitrogen was bubbled through to sweep out the last traces of the amine. The product was then extracted into ether and the resulting solution dried over $MgSO_4$.

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[1] Robert O. Lindblom, Electron-Spin-Resonance and Chemical Studies of Radiation Damage of Choline Chloride and Some of Its Analogs (Thesis), UCRL-8910, Oct. 1959, pp. 35-64.

N, N-dimethylaminoethanol-1-C^{14}

To the dry ether solution of the ethyl ester of N, N-dimethyl glycine, 57 mg of $LiAlH_4$ was added slowly with stirring. The reaction mixture was refluxed for 2 hours; then the excess $LiAlH_4$ was decomposed by the careful addition of moist methanol. The resulting mixture was evaporated to dryness and the product and all other volatile components were distilled into a trap cooled in liquid N_2. The residue remaining after the evaporation was washed twice with aqueous methanol and these washes were also distilled into the liquid N_2 trap.

$[(CH_3)_3N\text{-}CH_2C^{14}H_2OH]^+Cl^-$

To approximately one-half the ether-water-methanol solution of N, N-dimethylaminoethanol was added 0.25 ml of CH_3I, and the resulting solution was refluxed overnight. The reaction mixture was then evaporated to dryness. The crude product was dissolved in absolute ethanol and recrystallized from an ethanol-ether mixture. The yield of pure choline iodide was 92 mg (53.8% based on ethyl bromoacetate). The specific activity was found to be 0.85 mC per mmole. The elemental analysis was: C, 26.12% (calculated 25.98%); H, 6.26% (calculated 6.11%).

Because of the great radiation sensitivity of choline chloride, the choline was kept in the iodide form. For the radiation experiments, choline chloride of considerably lower activity was prepared. A typical conversion of some choline iodide to the chloride is as follows: To 5 mg of choline iodide-C^{14} dissolved in water excess AgO was added and the mixture was shaken briefly. The excess oxide and silver iodide was filtered off and the clear filtrate titrated to neutrality with 0.1 N HCl. To this solution was added approximately 8 grams of inactive choline chloride. The solution was evaporated to dryness and the crude choline chloride dissolved in absolute ethanol. After the product was recrystallized from an ethanol-ether mixture, the specific activity was found to be 0.28 μC/mmole. The elemental analysis was: C, 43.45% (calculated 43.01); H, 9.77% (calculated 10.11); Cl, 25.07 (calculated 25.41%).

Choline Chloride Irradiations

Samples of the crystalline C^{14}-labeled choline chloride were irradiated with the electron beam of a 3- to 5-Mev linear accelerator. The techniques used have been described previously.[2] The samples were given amounts of radiation sufficient to cause approximately 10% decomposition. Direct determinations of the amounts of decomposition were not made.

Acetaldehyde-C^{14} Degradations

After an irradiation, the sealed tube containing the choline chloride was opened and the contents dissolved in water at 0°. One mmole of inactive acetaldehyde was added as a carrier. The resulting solution was refluxed for 2 hours while the evolved acetaldehyde was swept by a helium stream

[2] R. M. Lemmon, P. K. Gordon, M. A. Parsons, and F. Mazzetti, J. Am. Chem. Soc. 80, 2730 (1958).

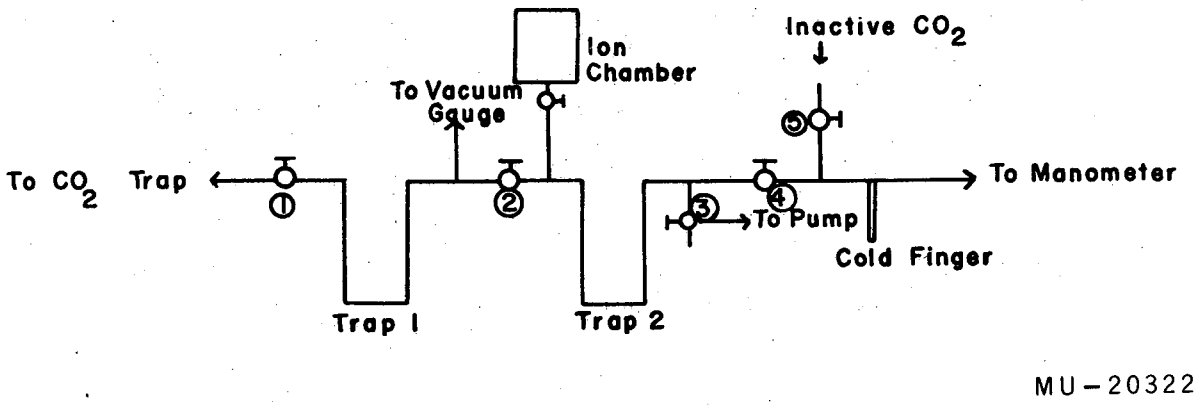

Fig. 13. Apparatus for transferring $C^{14}O_2$ into ion chamber.

In order to determine the radioactivity in the methylamine remaining after the Schmidt reaction, this compound was converted into its phenylisothiourea derivative in the following manner: The reaction mixture was cooled to 0° and made basic with 5 N NaOH. The resulting solution was heated and the liberated methylamine codistilled with approximately 1 ml of water into a small pear-shaped flask cooled in an ice bath. After the distillation, the flask was immediately transferred to dry ice—isopropanol bath and several grams of dry KOH added. The flask was then connected by a glass tube to another small pear-shaped flask containing 0.5 ml of phenyl isothiocyanate. The second flask was also cooled in a dry ice—isopropanol bath. Both flasks were then evacuated to less than 1 mm pressure and the cooling baths were removed. The system was allowed to come to room temperature slowly and stand overnight. The crystals that formed in the isothiocyanate flask, along with those precipitated by the addition of several ml of hexane, were filtered off, washed with hexane, and dried. A melting point was taken and the yield determined. A small portion was dissolved in a liquid scintillation solvent and the activity determined as described previously.[4] Another small sample was subjected to carbon and hydrogen analysis. Two samples of phenylmethylthiourea were prepared from the decomposed choline. Both gave very satisfactory elemental analyses, but one of them (sample No. 2) yielded a mixture of two kinds of crystals. One kind had the same melting point as recorded in the literature (113°); the other kind was lower melting. (See Table III.) In view of the good elemental analysis, it is highly probable that the lower-melting crystals are simply another crystalline form of the desired phenylmethylthiourea.

Table III

Elemental analyses and melting points of phenylmethylthiourea derivatives

		Found	
	Calculated	Sample 1	Sample 2
C (%)	57.79	57.59	57.57
H (%)	6.06	5.90	5.98
N (%)	16.85	17.10	--
S (%)	19.29	19.10	--
mp(°C)	113 (lit.)	112-114	112-114 92-94

[4] Elton M. Baker, in Chemistry Division Quarterly Report, UCRL-3157, September 1955, p. 51.

Results and Conclusions

Two samples of irradiated choline chloride were analyzed. The results of the acetaldehyde degradations are presented in Table IV.

The results of these experiments demonstrate that there is no symmetrical two-carbon species in the mechanism yielding acetaldehyde from irradiated crystalline choline chloride. The carbinol carbon atom of choline chloride is the aldehydic carbon in the acetaldehyde.

Table IV

Degradations of acetaldehyde from irradiated $[(CH_3)_3NCH_2C^{14}H_2OH]^+Cl^-$

Sample number	CO_2		$C_6H_5NHCSNHCH_3$		Activity in C-1 of acetaldehyde (%)
	Yield[a] (%)	Activity per mmole (10^{-2} μC)	Yield[a] (%)	Activity per mmole (10^{-5} μC)	
1 (100 mg)	50	4.07×10^{-2} μc	41	7.1×10^{-5} μc	99.8
2 (115 mg)	34	0.92×10^{-2} μc	28	4.8×10^{-5} μc	99.5

[a] Assuming a 10% decomposition of the choline chloride, providing about 0.1 mmole of $CH_3CHO\text{-}C^{14}$ added to 1.0 mmole of carrier acetaldehyde.

[b] The values in this column indicate that sample No. 1 was about 16% decomposed and sample 2 was about 4% decomposed.

PHOTOINDUCED ESR SIGNALS IN A CAROTENOIDLESS MUTANT OF RHODOPSEUDOMONAS SPHEROIDES

Gaylord Androes and Mary Singleton

Many experiments in this Laboratory have been concerned with the photoinduced electron spin resonance (ESR) signals from biological materials. In particular, we have been interested in such signals from photosynthesizing plants.[1] Although certain important properties of the observed signals have been established, it has not yet been shown that the photoinduced signal is intimately connected with the process of photosynthesis. One way of showing such a correlation might be to remove various substances known to be involved in photosynthesis from the plants, to see what effect this has on the observed ESR signal.

In following this approach we have taken advantage of the fact that some suitable photosynthetic materials can be made to exist in a mutant as well as a wild form. In Rhodopseudomonas spheroides, the case to be discussed, the wild type (R. S. w) has a full complement of pigment molecules, while the blue-green mutant (R. S. m) lacks the carotenoids. Since these pigments have been shown to be related to the photosynthetic process,[2] their absence could have an effect on the photoinduced signal. We have compared the photoinduced ESR signals arising in R. S. w and R. S. m as a function of various parameters.

Sample Preparation

The cell suspensions used were grown from slant cultures obtained from Professor R. Y. Stanier.[3] The blue-green mutant (obtained by ultraviolet irradiation of the wild type) was strain u. v. 31. R. S. bacteria grow anaerobically in the light. However, the mutant is killed when subjected to both light and oxygen, so, initially, the sample preparation was carried out in a dry box in the absence of oxygen. That is, after it was centrifuged in air, and in dim light or in the dark, the supernatant was removed, the pasty residue of bacteria painted on a metal rod, and the material on the rod dried in a nitrogen atmosphere. The sample was then transferred rapidly to the ESR cavity, where a nitrogen atmosphere was maintained during the various resonance experiments.[4] Low temperatures were maintained by passing cold nitrogen gas over the sample.

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[1] P. B. Sogo, M. Jost, and M. Calvin, Radiation Research, Supplement 1, 511 (1958).

[2] L. N. M. Duysens, Transfer of Excitation Energy in Photosynthesis (Thesis), Utrecht, 1952.

[3] All information concerning the physiological properties of R. S. w and R. S. m. (blue-green) was obtained from W. R. Sistrom, Mary Griffiths, and R. Y. Stanier, J. Cell. Comp. Physiol. 48, 473 (1956), and M. Dworkin, J. Gen. Physiol. 41, 1099 (1958).

[4] For a more complete description of the sample and cavity arrangement, see P. B. Sogo, N. Pon, and M. Calvin, Proc. Natl. Acad. Sci., 43, 387 (1957).

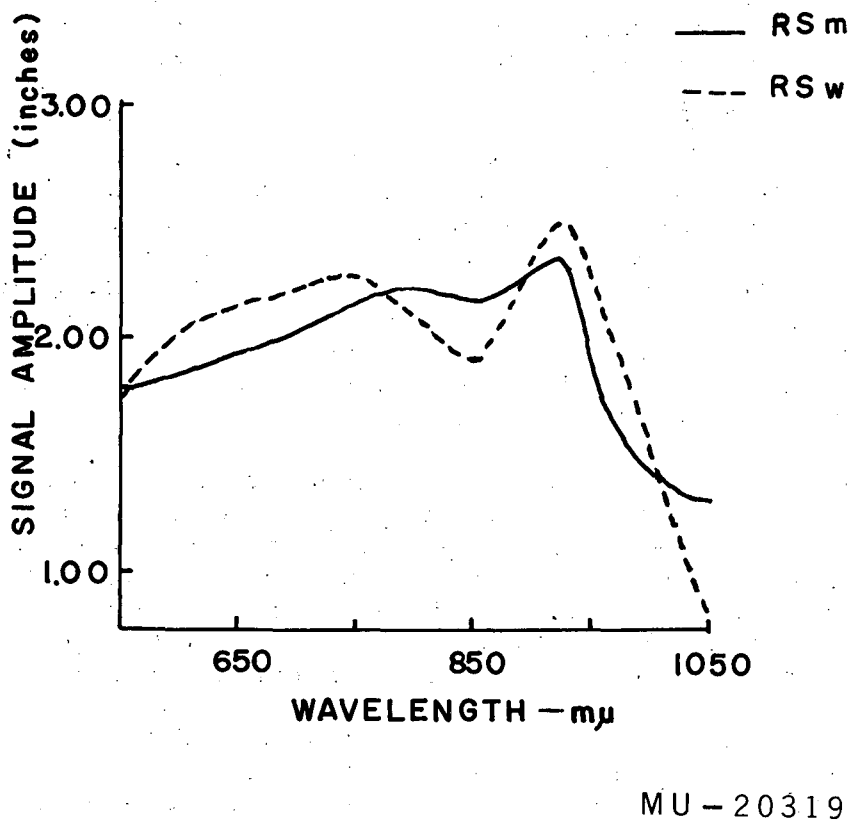
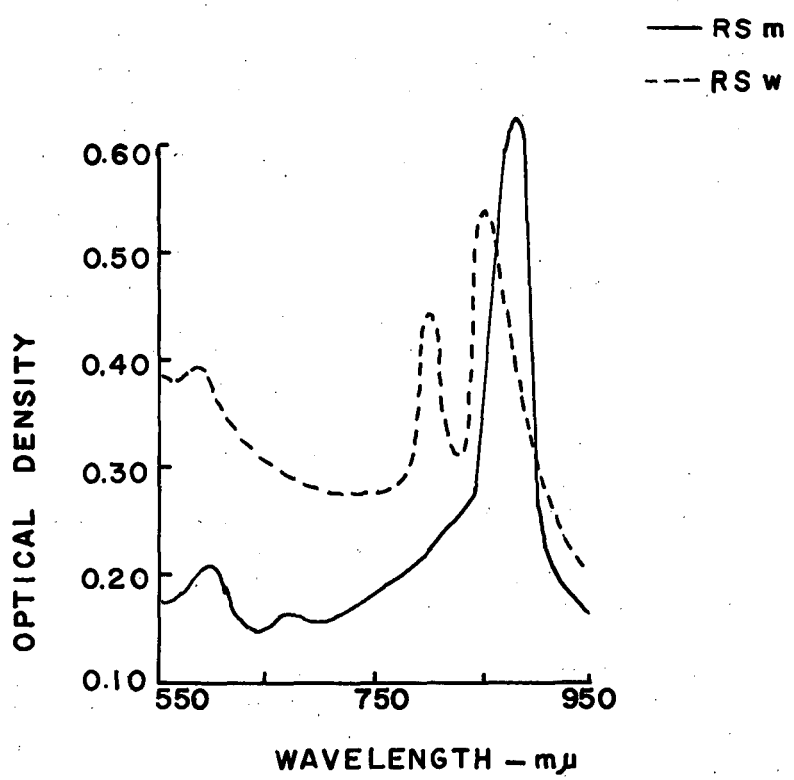

RS m
RS w
SIGNAL AMPLITUDE (inches)
3.00
2.00
1.00
650
850
1050
WAVELENGTH — mμ
MU-20319

Fig. 14.



MU-20320

Fig. 15.

Discussion

In the blue-green mutant of R. S. the carotenoid pigments are missing. However, there are similar molecules in their place; e. g., C_{40} polyenes or phytoenes. Phytoenes differ from carotenoids mainly in that they lack the conjugated π system of the latter. This absence of conjugation makes the phytoene much less rigid than carotene, but apparently has little effect on the cell structure. Stanier et al. believe that the intrinsic effectiveness of the chlorophyll in the mutant as a photosynthetic pigment is approximately the same as in the wild type. Thus, one would expect differences between the mutant and the wild type to be due to the absence of carotenoids, and not to deficiencies in the remaining photosynthetic apparatus.

The only difference noted between the behavior of the R. S. m and R. S. w photoinduced ESR signals is in the action spectra. This difference is believed to be largely due to the differences in optical absorption coefficients (Fig. 15). The difference in absorption coefficient reflects the absence of carotenoids in the mutant, but its effect in our experiments is to determine the effective sample thickness by fixing the depth of light penetration.

Within the accuracy of our experiments other differences were not found. This indicates either that the carotenoids have no function in the formation and eventual decay of the photoinduced spin signals, or, if they have some function, the phytoene is equally efficient in carrying it out. No inference can be drawn concerning a possible intimate relation of the unpaired electrons to the photosynthetic process.

In view of the observation that the mutant is susceptible to photokilling, in which one or more of its constituents (including chlorophyll) is oxidized, it is reasonable that the ESR signal in the mutant should react to light and oxygen. It is strange, however, that the same reaction is found in the wild type when it is subjected to the same conditions, for the wild type is not photokilled. One must conclude that in the ESR experiment, and within our accuracy, the photokilling effects are secondary.

It is probable that enhanced ESR spectrometer sensitivity would bring out several differences between R. S. w and R. S. m. For example, differences might be found in the photosignal rise times, and in the initial fast decay times, as well as in the effects of photokilling on the mutant. A more sensitive spectrometer is planned, and these bacteria will probably be further studied after it is put into operation.

STUDIES ON THE OCCURRENCE OF MANGANESE PHAEOPHYTIN IN PLANTS

Edward Markham

The initial stage of the photosynthetic cycles 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 light energy to a receptor molecule; the actual energy conversion would then be carried out by the receptor molecule. Bourdon[2] 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. He proposed the following scheme for iron (P = phaeophytin):

$$h\nu + P_{Mg}II \longrightarrow P^*_{Mg}II\,,$$

$$P^*_{Mg}II + P_{Fe}II \longrightarrow P_{Mg}II + P^*_{Fe}II\,,$$

$$P^*_{Fe}II \longrightarrow P_{Fe}III + e^-\,.$$

According to this scheme the excited chlorophyll would pass on its energy to the iron phaeophytin (in the ferrous state), which would then evolve an excited electron. The ferric phaeophytin would then be reduced back to the ferrous state by the abstraction of an electron from a molecule of water, while the excited electron would be transferred to an electron acceptor (thus furnishing the chemical energy necessary for the photosynthetic cycle). It can be seen that such a compound need be present only in trace amounts. Bourdon's results did in fact show that an iron phaeophytin was present. However, he obtained no evidence to suggest it functioned in the manner expected. It was decided to reinvestigate this problem, considering manganese phaeophytin as a receptor molecule instead of iron phaeophytin. This compound, in the light of recent work on manganese phthalocyanine, afforded an even more elegant explanation of the energy conversion. Manganese can exist in a divalent and tetravalent state. Consider the scheme

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[1] J. A. Bassham and M. Calvin, in Chemistry Division Quarterly Report, UCRL-2853, Feb. 1955.

[2] Jean Bourdon, (UCRL), Iron Chlorophyll Compounds in Green Plants, unpublished final report, Sept. 1956.

$$h\nu + P_{Mg}^{II} \longrightarrow P^{*}_{Mg}{}^{II} ,$$

$$P^{*}_{Mg}{}^{II} + P_{Mn}^{II} \longrightarrow P_{Mg}^{II} + P^{*}_{Mn}{}^{II} ,$$

$$P^{*}_{Mn}{}^{II} + H_2O \longrightarrow P_{Mn}^{IV}{}_{0} + 2H^{+} ;$$

$$P_{Mn}^{IV}{}_{0} + h\nu \longrightarrow P_{Mn}^{II} + 1/2\ O_2 .$$

It can be seen that this is a possible explanation of not only the energy conversion but also the mechanism of photolysis. The possibility of this scheme is the object of the following work. The problem resolves itself under three headings:

(a) Is manganese present in the photosynthetic centers and is it in the necessary concentration and molecular combination?

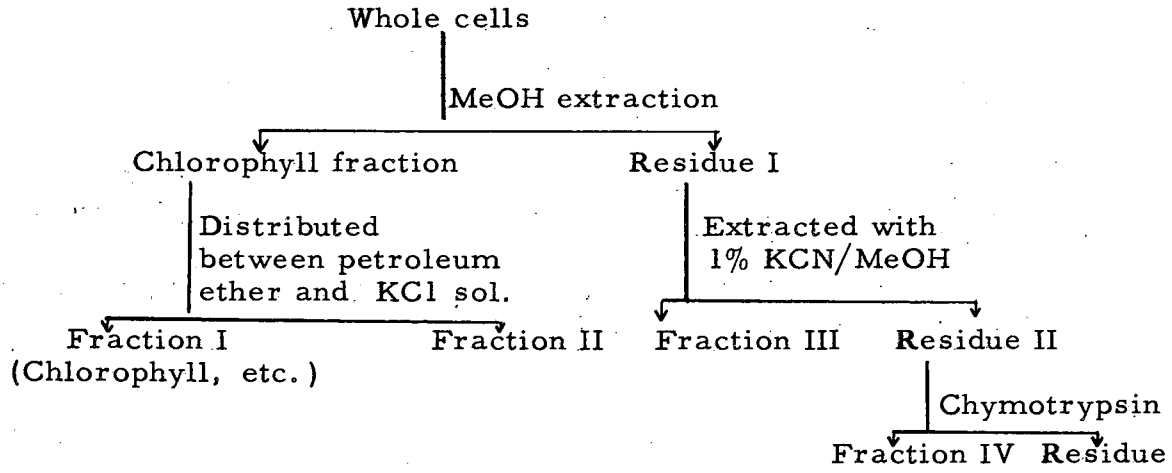
(b) Can manganese phaeophytin combine reversibly with oxygen and photolyze water? That is, is the reaction sequence chemically feasible?

(c) If (a) and (b) are satisfied, then a means must be found to show that the mechanism operates in vivo.

Investigations on the Presence of Manganese in Plant Material

Whole chloroplast preparations were sonically ruptured and the fragments containing chlorophyll were centrifuged. These fragments were ashed and the nature of the metallic content of the ash determined by emission spectrographic analysis. It was found that only two transitional metals were present in the ash, iron and manganese. The ratio Mg/Mn was about 800 and there was approximately fifteen times as much iron as manganese. The large amount of iron may be attributed to cytochromes.

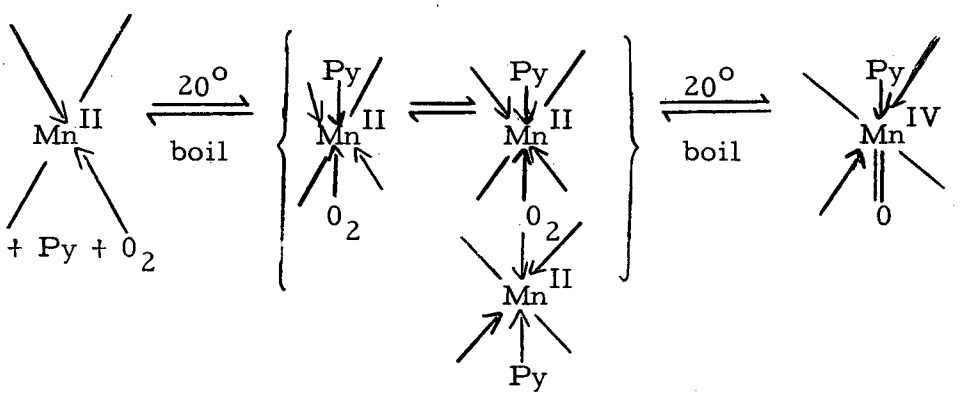
Chlorella were grown in a manganese-deficient medium and Mn^{54} was introduced in the divalent form. The cells were centrifuged, washed, and fractionated as follows:



Virtually all the activity was found in Residue I. A third of the activity could be extracted from this residue by means of 1% KCN/MeOH solution. The two-thirds remaining activity was released from Residue II by partial digestion with chymotrypsin. Thus, it appears that two forms of manganese are present, either by virtue of different bonding of the simple Mn^{II} ion or by its being present in different compounds. Further work is required to decide between these two alternatives. However, that manganese is strongly bonded in Residue I, which is proteinaceous and is released by KCN/MeOH and hydrolysis, suggests a manganese cytochrome system. Cytochromes are known to be strongly bonded to proteins. However, the presence of manganese cytochrome is only a surmise.

Investigations of the Chemical Feasibility of the Mechanism Using Model Compounds

Elvidge and Lever[3] have reported that manganese II phthalocyanine, when dissolved in pyridine, absorbs oxygen. Half a molecule of oxygen is absorbed per molecule of manganese phthalocyanine. The reaction is reversible since, if the solution is boiled, manganese II phthalocyanine is reformed; the royal blue solution of the oxygen compound reverts to the olive green color of the parent compound. It was not discovered whether oxygen is evolved or pyridine-N-oxide is formed. Elvidge and Lever proposed the following scheme for these reactions (where Py = pyridine):

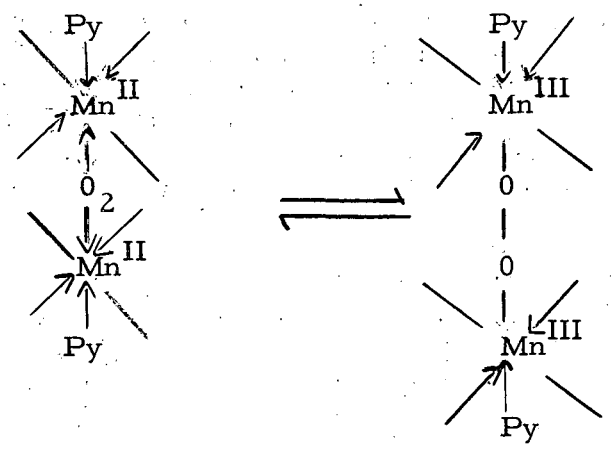


(1) (2) (3) (4)

This scheme resembles somewhat the proposed scheme for the energy transformation by chlorophyll and photolysis mechanism. It was decided to investigate this reaction further, especially with respect to possible photochemical effects.

[3] J. A. Elvidge and A. B. P. Lever, Proc. Chem. Soc. June/July, 195 (1959).

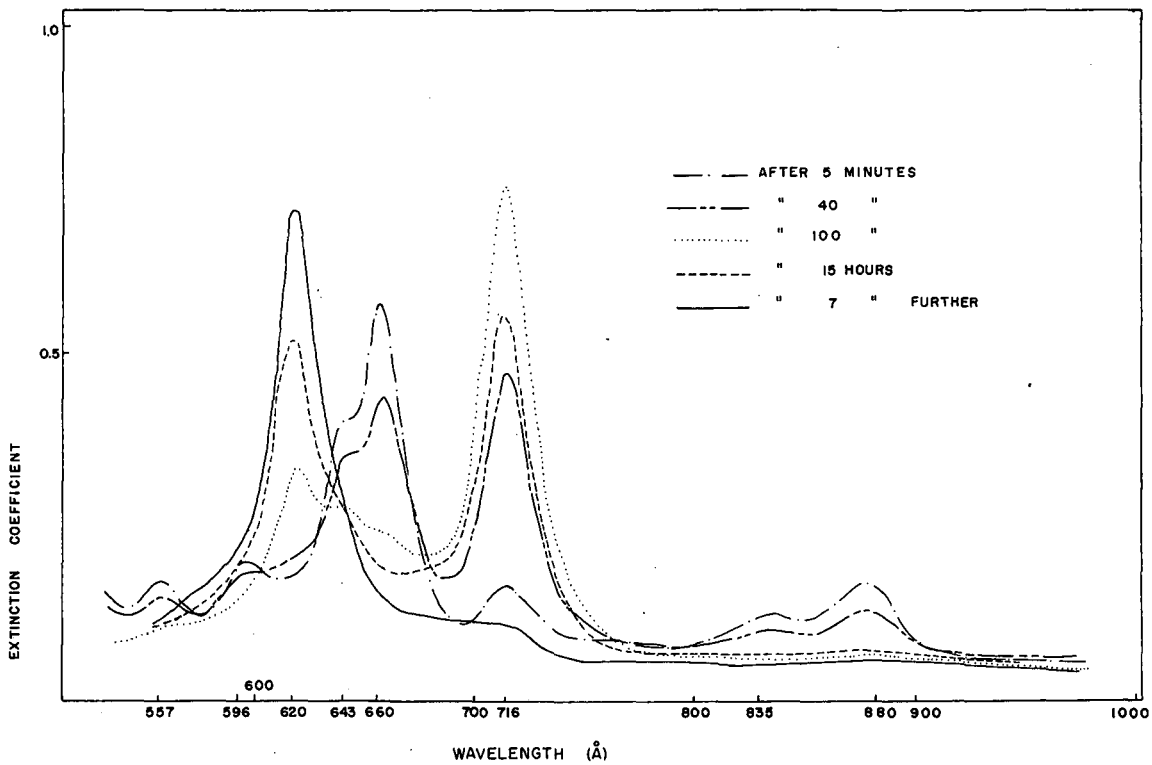
Manganese II phthalocyanine (structure 1) was prepared by standard methods[4] and purified by sublimation at 400°/0.5 cm Hg. It formed long black needles. These crystals were suspended in pyridine and allowed to stand several days exposed to air. The manganese II phthalocyanine gradually went into solution until the solution eventually deposited purple rhombs of manganese IV pyridine phthalocyanine oxide (structure 4 above). Both compounds gave the correct analysis for the assigned structures. However, when visible spectra were taken it was found that a discrepancy was apparent in the work of Elvidge and Lever. These workers reported that the spectrum of manganese II phthalocyanine in pyridine contained a band at 7125 A. We found, on repeating their work, that a band did occur in this region but that it changed in intensity with time. It was concluded that the band at 7125 A was probably the Mn II compound (3) in the trivalent peroxide form (structure 5), as illustrated below, formed by atmospheric oxidation:



(3) (5)

Manganese II phthalocyanine was dissolved in pyridine under nitrogen. The resulting olive green solution was then examined spectrophotometrically at regular intervals while exposed to air. A series of absorption curves was obtained (Fig. 16) which showed quite definitely that a transient intermediate was formed during the reaction. This species had an absorption band at 716 mμ and probably corresponded with Elvidge and Lever's band at 712 mμ. It was not, however, manganese II phthalocyanine, since the absorption gradually built up to a maximum and then decreased until it was no longer apparent. The band at 620 mμ belonged to the manganese IV pyridine phthalocyanine oxide (as known from our pure specimen). Thus, all the other bands that decreased in absorption while the 716- and 620-mμ bands increased were attributed to manganese II phthalocyanine, i.e., the bands at 880, 835, 660, 643, 596, and 557 mμ. This experiment was repeated, with spectra taken at regular intervals after the spectral cell had been kept alternately in the light and dark. It was found that light had a profound effect on the rate of oxidation; the reaction was slow in the dark and rapid when illuminated

[4] P. A. Barret, R. E. Dent, and R. P. Linstead, J. Chem. Soc. 1719 (1936).



MU-20502

Fig. 16.

with sunlight. This effect is illustrated in Fig. 17, where the curves close together were obtained from solutions both before and after the solutions were kept in the dark.

In order to study further photochemical effects of this reaction, a special cell was constructed (Fig. 18). The cell consisted of a small flask of about 15 to 20 ml capacity connected by a side arm to a 1-cm cell. Both vessels were stoppered, and the stopper of one carried a stopcock. Manganese II phthalocyanine was introduced into the spectral cell and pyridine into the flask. The pyridine was frozen in liquid nitrogen and the apparatus evacuated on a high-vacuum line. The stopcock was then closed and the pyridine allowed to thaw; it was then frozen and re-evacuated. This process was repeated three times. The pigment was then dissolved in the pyridine in the dark overnight. The following day, when solution was complete and the system at equilibrium, the spectrum was taken. The vessel was submerged in water and exposed for 15 minutes to sunlight, and then the spectrum was redetermined. It was found that even under high vacuum sufficient oxygen is present to allow all three species to be present. After the solution has been kept in the dark all three species can be detected in the spectra. However, on exposure of the solution to sunlight, the 716-mμ band disappears while the 835- and 880-mμ bands increase (Fig. 19). After the solution has been kept in the dark overnight the 716-mμ band re-forms and the 835- and 880-mμ bands decrease. It appears, then, that the peroxide (structure 5) is photochemically broken down to manganese II phthalocyanine (structure 1). That the re-formation is never complete (note in Fig. 19 that the bands never reach their former value again) suggests that the peroxide also breaks down, but more slowly, to manganese IV pyridine phthalocyanine oxide (structure 4).

In order to see if this was the case, manganese II phthalocyanine was dissolved in pyridine to form a dilute solution. The solution was exposed to air and the oxidation observed spectroscopically. When the band at 716 mμ was well formed, the vacuum cell was evacuated in the manner described, thus "fixing" the system. The vacuum cell was kept overnight in the dark to allow equilibrium to be attained.

The following day the spectrum was taken before and after exposure to sunlight. It was found (Fig. 20) that the 716-mμ band disappeared and the 620-mμ band increased, as expected. However, a small decrease in intensity of the bands at 835 and 880 mμ occurred, but the 660-mμ band showed a definite increase. These bands belong to the manganese II phthalocyanine, which would also be expected to increase. That two of them did not increase was attributed to variations in the spectrophotometer and to the fact that the solution was so dilute that it did not permit changes in the 835- and 850-mμ bands to be seen. The system showed no further change after being kept in the dark overnight. This was to be expected from the foregoing argument, since if the reaction that was reversible could not be observed to proceed one way, it certainly could not the other.

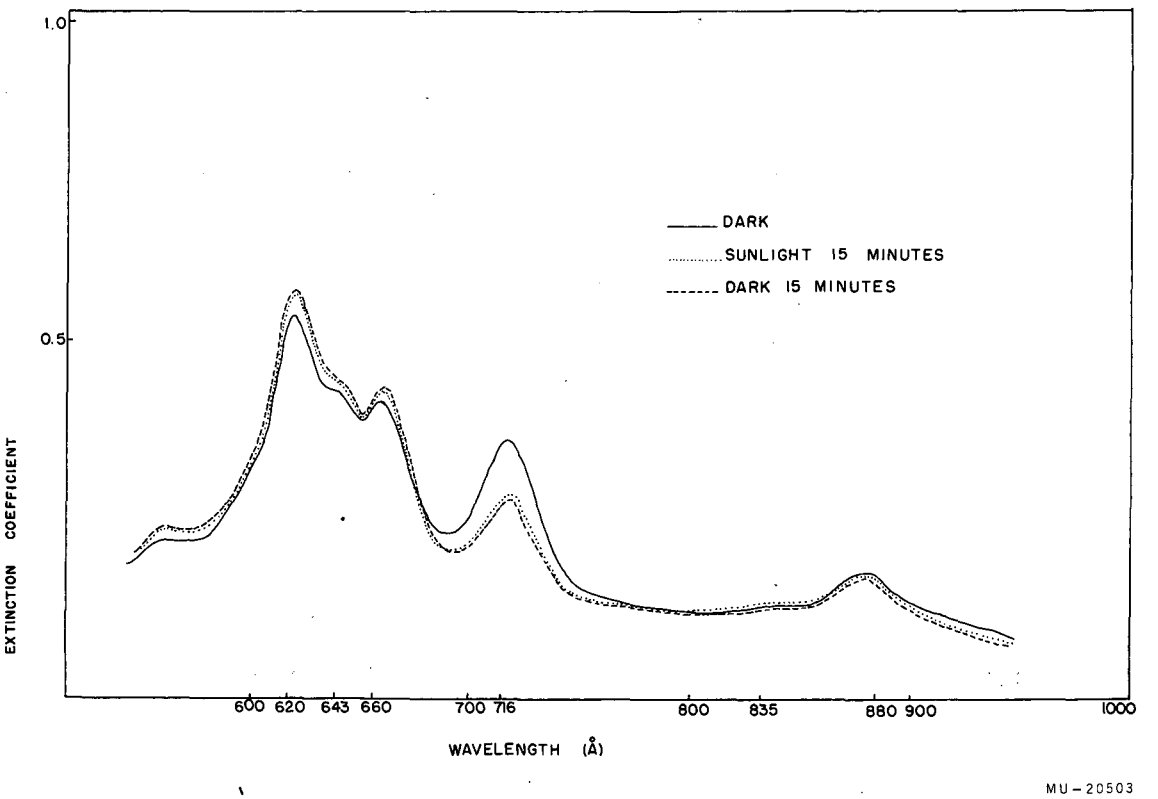

1.0
0.5
EXTINCTION COEFFICIENT
DARK
SUNLIGHT 15 MINUTES
DARK 15 MINUTES
600 620 643 660
700 716
800
835
880 900
1000
WAVELENGTH (Å)
MU-20503

Fig. 17.

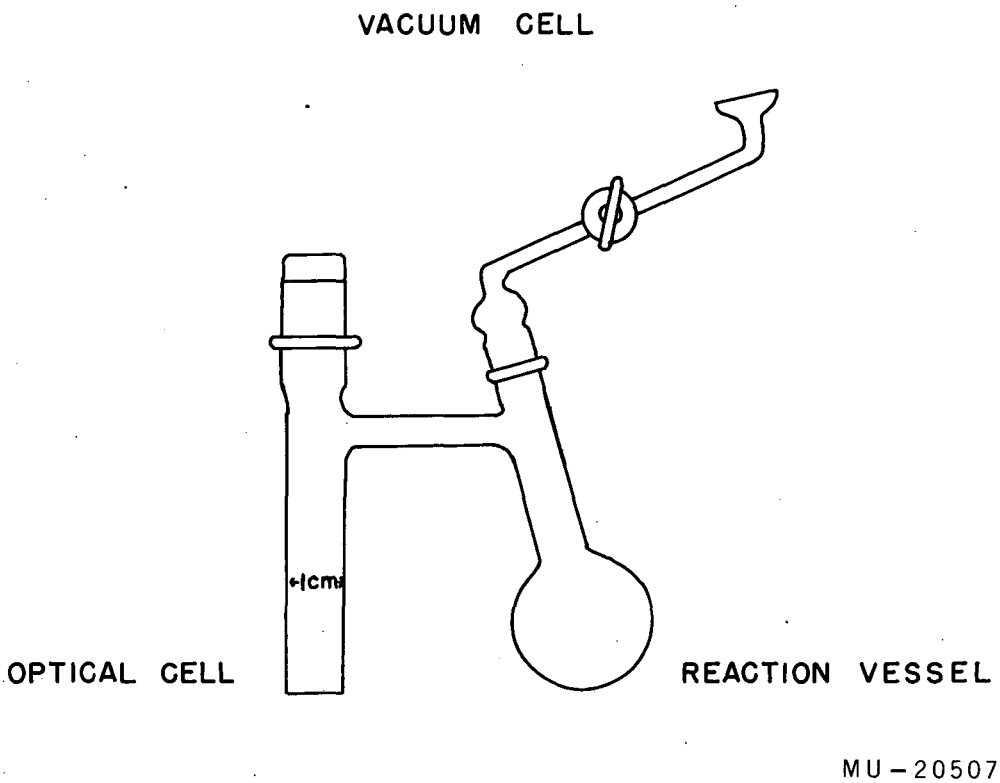


Fig. 18. Apparatus for illumination of Mn^{II} phthalocyanine.

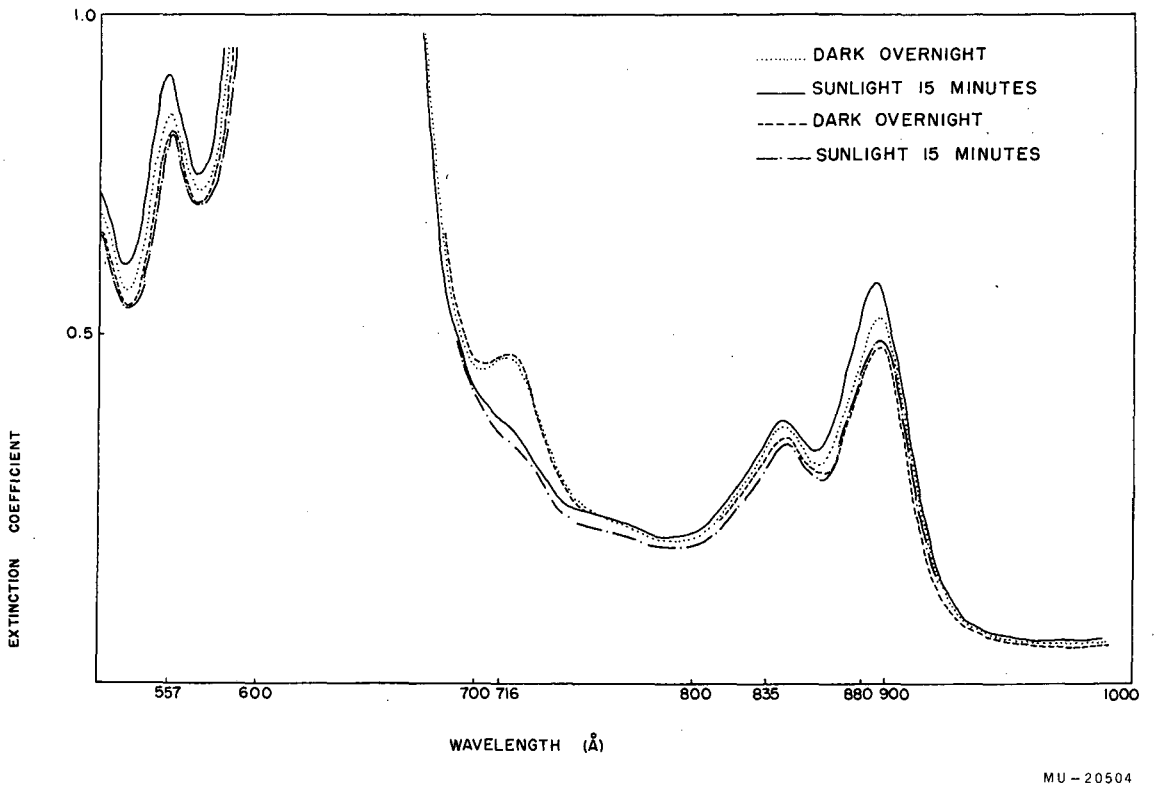

1.0
0.5
EXTINCTION COEFFICIENT
DARK OVERNIGHT
SUNLIGHT 15 MINUTES
DARK OVERNIGHT
SUNLIGHT 15 MINUTES
557
600
700 716
800
835
880 900
1000
WAVELENGTH (Å)
MU-20504

Fig. 19.

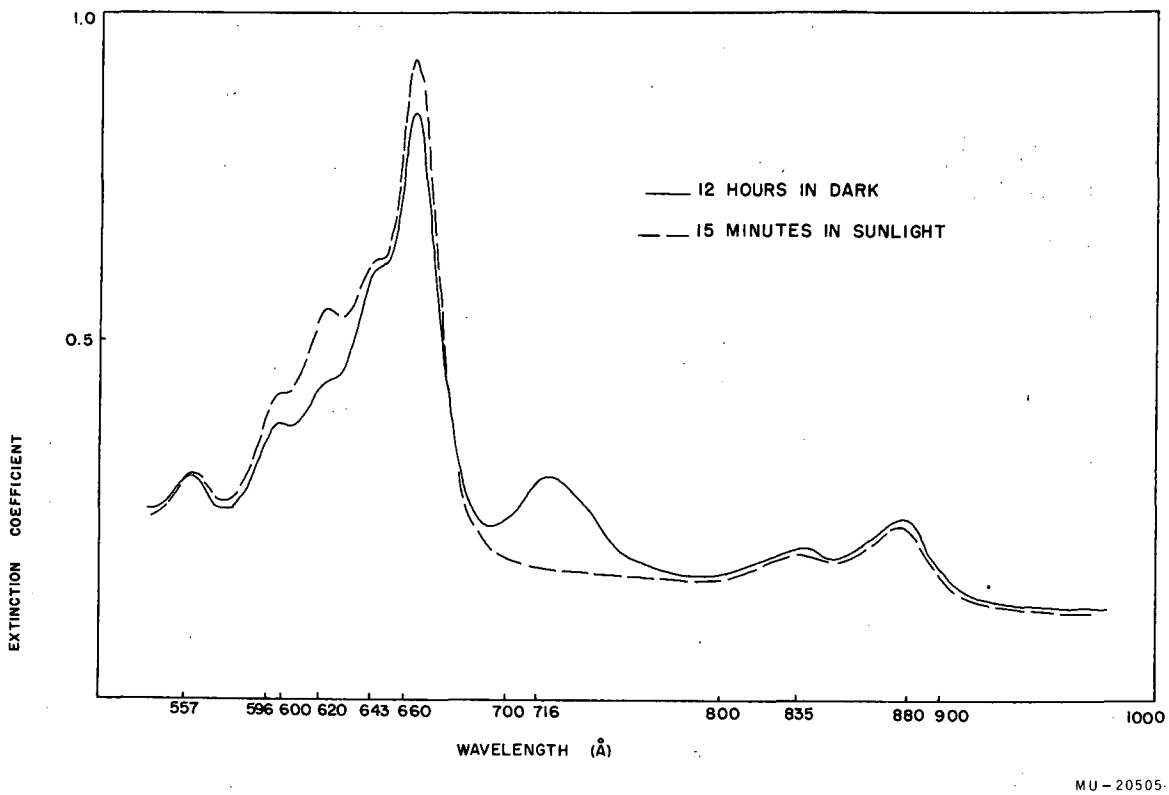
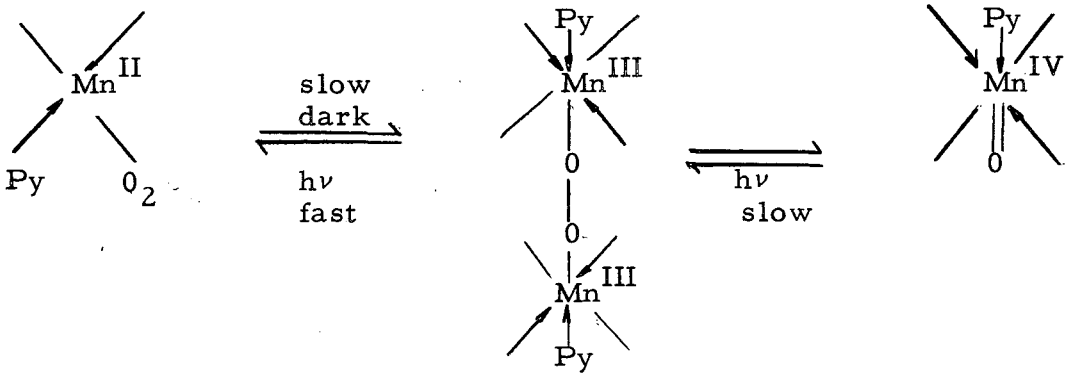

1.0
0.5
EXTINCTION COEFFICIENT
12 HOURS IN DARK
15 MINUTES IN SUNLIGHT
557
596 600 620 643 660
700 716
800
835
880 900
1000
WAVELENGTH (Å)
MU-20505

Fig. 20.

It seemed from these results that the formation of manganese IV pyridine phthalocyanine oxide was irreversible and that the compound was photochemically decomposed to simpler fragments showing no absorption in the regions examined. This supposition was tested by making a solution of the compound in pyridine and taking its spectrum. The cell was evacuated and kept in the dark overnight. The next day no apparent change in spectrum was observed. On successive exposures of the solution to sunlight, the single band at 620 mμ decreased in intensity without the appearance of any new bands (Fig. 21).

The conclusions of this work, as it stands at present, are summarized as follows:



Thus the formation of the peroxide at low pressures is a slow reaction proceeding in the dark. At high oxygen pressures, by the Law of Mass Action, it becomes fairly rapid. The peroxide is broken down by light to form, mainly, manganese II phthalocyanine and a little manganese IV pyridine phthalocyanine oxide at low pressures. At a high oxygen pressure the reaction favors the formation of the oxide. No evidence has yet been found to suggest that the formation of the oxide is reversible at room temperature. This is still under investigation.

Possible Methods of Detecting Manganese Compounds in vivo

Should the foregoing work show that the postulated mechanism for photolysis is possible, it will be necessary to demonstrate it *in vivo*. This would entail the identification of minute amounts of manganese compounds and the determination of their various valence states.

The use of Mn^{54} has already shown the presence of manganese, and this work is being pursued in the hope that manganese concentrates may be obtained for further study. However, such a method will not allow the determination of the various valence states. Electron spin resonance has been selected as the method of choice for that determination. The technique is extremely sensitive and requires only minute amounts of material. Three model compounds of manganese have been studied by this method: manganese II phthalocyanine, manganese IV pyridine phthalocyanine oxide, and manganese II bis-(3, 3', 4, 4', 5, 5'-hexamethyldipyrromethene). The latter compound was prepared by treatment of an ethanolic solution of 3, 3', 4, 4', 5, 5'-hexamethyldipyrromethene with an ammoniacal solution of manganous chloride. It has the following structure:

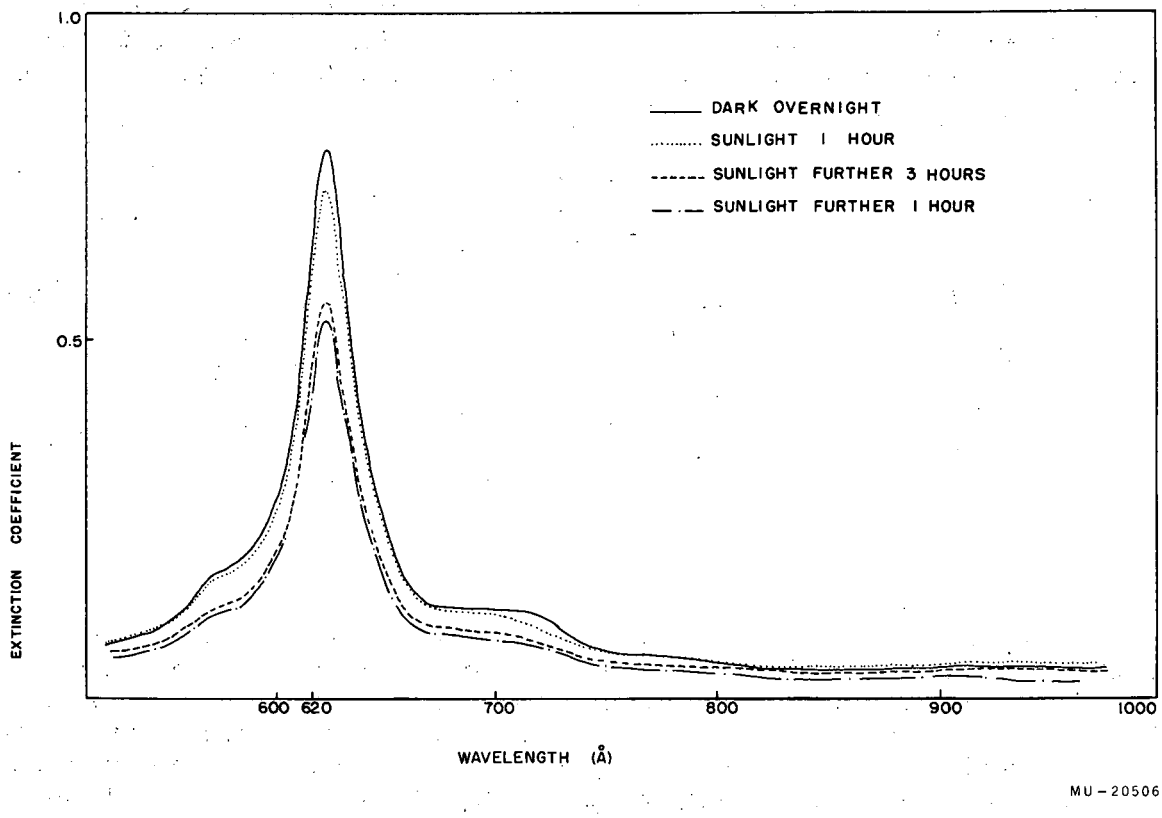
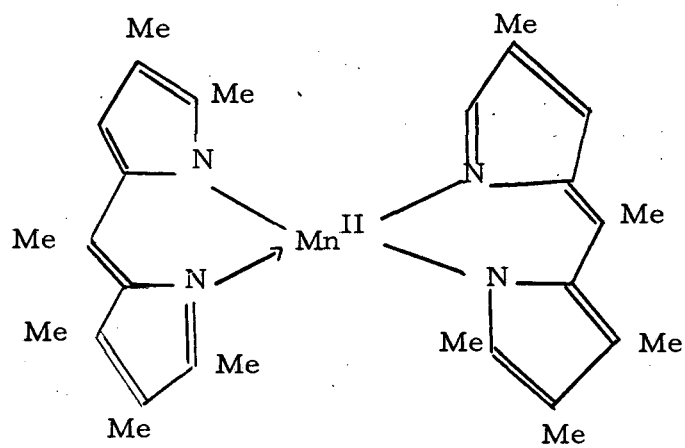

1.0
0.5
EXTINCTION COEFFICIENT
DARK OVERNIGHT
SUNLIGHT 1 HOUR
SUNLIGHT FURTHER 3 HOURS
SUNLIGHT FURTHER 1 HOUR
600
620
700
800
900
1000
WAVELENGTH (Å)
MU-20506

Fig. 21.



These three compounds afforded manganese in a variety of valence states, i.e., manganese II phthalocyanine is square planar (dsp^2), manganese IV pyridine phthalocyanine oxide is octahedral (d^2sp^3), and manganese II bis-3, 3', 4, 4', 5, 5'-hexamethyldipyrromethene is tetrahedral (sp^3). The compounds have been studied by ESR with little success. At room temperature and at 100°K all have given a broad resonance over 3000 gauss with no fine structure. A restudy will be made when the ESR apparatus has been modified to increase its resolution.

Future Work

Throughout this report, the nature of work to be carried out in the near future has been indicated. However, one important facet remains that has not been studied at all. In the proposed photolytic scheme (page 41) that following reaction is postulated:

$$P^{*}Mn^{II} + H_2O \longrightarrow P\,Mn^{IV}O + 2H^{+}\,.$$

So far no evidence for this reaction has been found. Experiments are to be made using solutions of manganese II phthalocyanine in aqueous pyridine in an attempt to obtain some verification of this step.

Electrical Properties of Organic Solids. Part III

EFFECTS OF ADDED ELECTRON ACCEPTORS ON THE ELECTRICAL AND MAGNETIC PROPERTIES OF METAL-FREE PHTHALOCYANINE, VIOLANTHRENE, TETRACENE, AND PERYLENE

David R. Kearns

The effect of the strong oxidizing agent (electron acceptor) ortho-chloranil on the electrical and magnetic properties of films of metal-free phthalocyanine has already been discussed in detail elsewhere.[1, 2] In this report, the effects of two additional electron acceptors, iodine and tetra-cyano ethylene, on the electrical and magnetic properties of metal-free phthalocyanine as well as of violanthrene, perylene, and tetracene are discussed.

The dark- and photoconductivity of the pure and doped organic compounds was measured in a sample cell prepared as follows. The organic compound to be studied was sublimed onto a glass slide to which graphite electrodes had been applied. This is shown in Fig. 22. The illumination was carried out by focusing light either on the face of the sample with the electrodes or on the back. Dark- and photocurrent measurements were carried out in the manner described previously.[1, 2, 3] Ortho-chloranil as a 10^{-5}-mole/cc benzene solution was applied by spraying onto the back of a surface cell. Tetra-cyano ethylene was also applied in this manner, while iodine was simply sublimed onto the back of a surface cell. In all cases the geometry of the surface cells was such that the measuring electrodes were separated from the "doping" agents by a thick layer (5×10^{-4} cm or greater) of organic dye. (See Fig. 22.) For the electron spin resonance (ESR) measurements, the organic dyes were sublimed onto 0.5-cm-wide strips of blank glass which could be placed into the spin-resonance spectrometer cavity. A hole in the end of the cavity allowed the sample to be illuminated while it was in the cavity. A description of the spectrometer may be found elsewhere.[2] In certain experiments, band-pass filters were used to restrict the illuminating light to desired wave lengths.

The number of unpaired electron spins in the various systems was calculated by comparing their ESR absorption with that of a standard sample of 1, 1-diphenyl-2-picrylhydrazyl. The numbers quoted in this report are only approximate and, at best, good only within an order of magnitude.

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[1] D. R. Kearns, G. Tollin, and M. Calvin, J. Chem. Phys. 32, 1020 (1960). and [illegible]

[2] David R. Kearns, Electrical Properties of Organic Solids (Thesis), UCRL-9120, March 1960.

[3] G. Tollin, D. R. Kearns, and M. Calvin, J. Chem. Phys. 32, 1013 (1960). and [illegible]

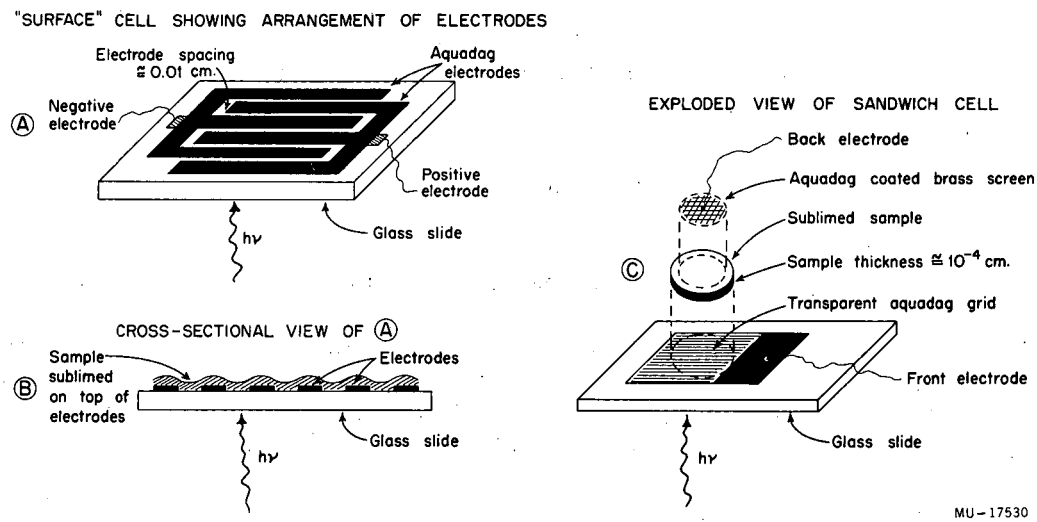

Fig. 22. Apparatus used for conductivity samples.

Results

A. Metal-Free Phthalocyanine—Ortho-chloranil System

1. Conductivity

The addition of 10^{-5} mole of ortho-chloranil to a surface cell of metal-free phthalocyanine produces an immediate increase (< 30 sec) of 2.5×10^5 in the dark conductivity of the phthalocyanine layer. Concomitantly, the photoconductivity is increased by a factor of 8×10^3. These results are discussed in detail elsewhere.[1,2]

2. Electron-Spin Resonance

The addition of ortho-chloranil to a layer of metal-free phthalocyanine produces a large concentration of unpaired electron spin ($\approx 0.3\%$) which is reversibly decreased by light strongly absorbed by phthalocyanine. A wide variety of earlier experiments has led to the conclusion that the same photoprocess results in the increase in conductivity and the decrease in unpaired electron-spin concentration.

B. Metal-Free Phthalocyanine—Iodine System

1. Conductivity

When a surface cell was exposed to iodine vapor, the dark conduction was increased by a factor of 5×10^4 and the photoconduction was increased by a factor of 3×10^4. Initially the increases in conductivity are larger by a factor of 10; however, there is apparently loss of iodine with time, since the measurements were carried out in air. The values reported are those obtained after several days, at which time the rate of iodine loss appears to be negligible.

2. Electron-Spin Resonance

The application of iodine to the surface of a phthalocyanine film produced an unpaired-electron-spin concentration of about 0.3% that reversibly decreased when the sample was illuminated. Presumably, the photoinduced increase in the conductivity and the photoinduced decrease in unpaired spin concentration are a result of the same process. However, this must be checked by measurement of the temperature dependence, spectral response, and kinetics of the two effects.

C. Metal-Free Phthalocyanine—Tetra-Cyano Ethylene System

1. Conductivity

The dark conductivity of a metal-free phthalocyanine film was increased by a factor of 10^3 by the addition of 10^{-4} mole of tetra-cyano ethylene to its surface. In addition, the phthalocyanine photoconductivity was increased by a factor of 4×10^4.

2. Electron Spin Resonance

The addition of 10^{-4} mole of tetra-cyano ethylene to a phthalocyanine film produces a large concentration of unpaired electron spins (1/2% based upon total number of phthalocyanine molecules). A reversible 30% decrease in this spin concentration was observed when the treated sample was illuminated. This is shown in Fig. 23.

D. Violanthrene—Ortho-Chloranil System

1. Conductivity

The addition of 10^{-5} mole of ortho-chloranil to a film of violanthrene increases the dark conductivity by a factor of 9×10^{2} and the photoconductivity by a factor of 1.5×10^{2}.

2. Electron Spin Resonance

A large concentration of unpaired electron spins corresponding to almost 100%, based upon the total number of violanthrene molecules present, was produced when 10^{-5} mole of ortho-chloranil was added to a violanthrene film. This spin concentration was increased reversibly by 4% when the sample was illuminated in the ESR spectrometer. This effect is shown in Fig. 24.

E. Tetracene—Ortho-Chloranil System

1. Conductivity

The addition of 10^{-5} mole of ortho-chloranil to a surface of tetracene produces a 50-fold increase in the dark conductivity of the tetracene layer and a 2×10^{2}-fold increase in the photoconductivity.

2. Electron-Spin Resonance

ESR absorption has been observed in this system. However, photo effects in the unpaired spin concentration have not been investigated.

F. Tetracene—Iodine System

1. Conductivity

The effect of iodine on tetracene conductivity has not yet been investigated.

2. Electron-Spin Resonance

When iodine was sublimed onto the surface of a tetracene film, a large ESR absorption was observed (10% based on the number of tetracene molecules present). Illumination of this sample resulted in a reversible 50% decrease in the unpaired spin concentration. This effect is shown in Fig. 25.

G. Perylene—Ortho-Chloranil System

1. Conductivity

The addition of 10^{-5} mole of ortho-chloranil to a surface cell of perylene produces a 4×10^{2}-fold increase in the dark conductivity of the perylene layer and an increase of 10^{3} in the photoconductivity.

2. Electron-Spin Resonance

ESR absorption corresponding to 0.1% can be observed when ortho-chloranil is added to a perylene film. Photo-spin resonance measurements have not yet been carried out.

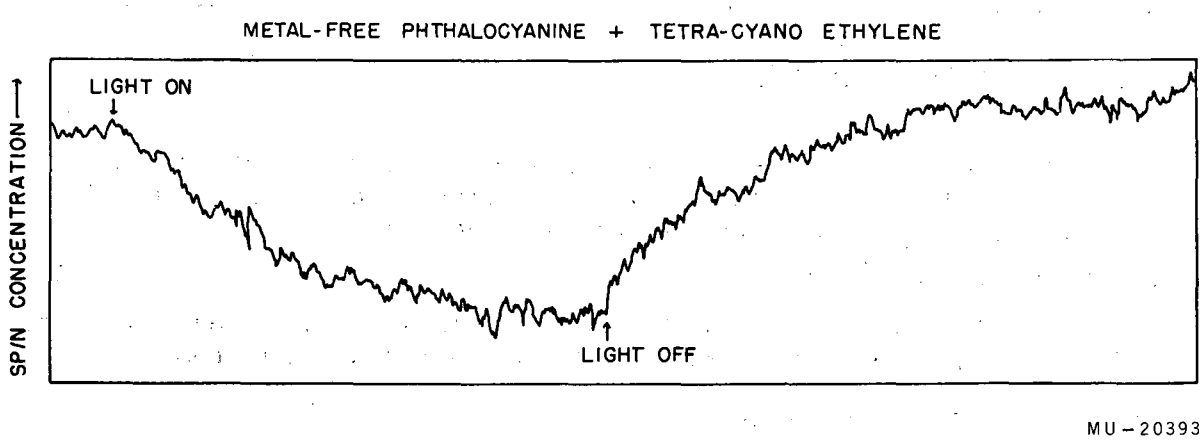

Fig. 23. Effect of illumination on spin concentration: metal-free phthalocyanine and tetra-cyano ethylene.

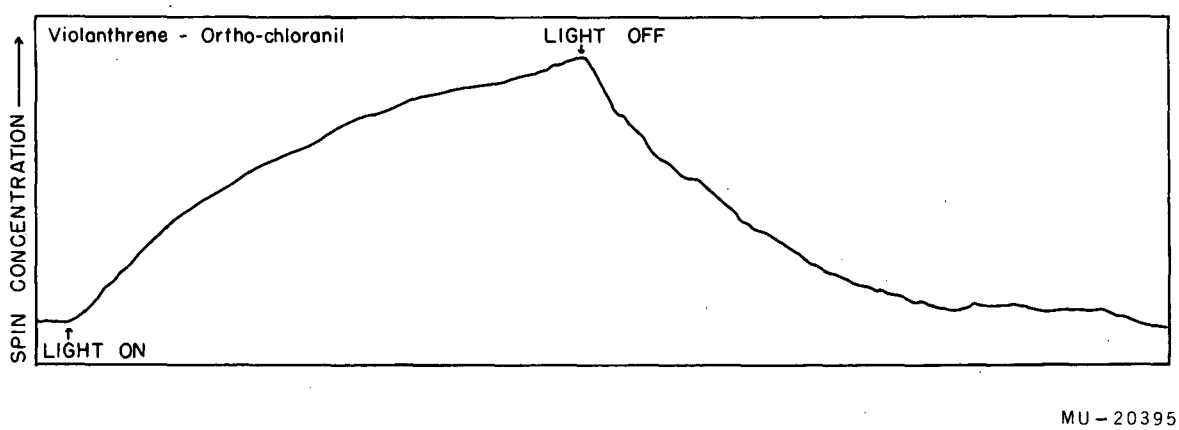

Fig. 24. Effect of illumination on spin concentration: o-chloranil and violanthrene.

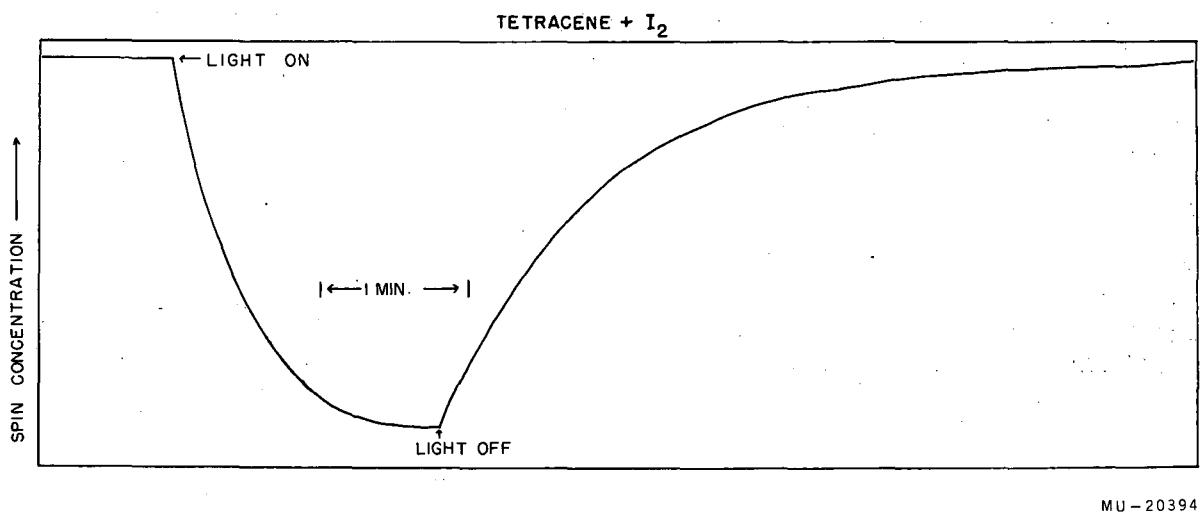

Fig. 25. Effect of illumination on spin concentration: tetracene and iodine.

The results of the conductivity and ESR measurements on the various systems are presented in Table V, along with the solid-state ionization potentials of the pure dyes. If one considers the effect of orthochloranil on the dark conductivity of the various dyes, there appears to be an inverse relationship between the increase in conductivity and the solid-state ionization potential. The results presented here will be further discussed later.

Table V

Results of conductivity and ESR measurements

Compound (and ionization potential, in ev)	Measurement	Pure material (amp)	Increase in conductivity due to the additive		
			10^{-5} M o-chloranil	10^{-4} M tetra-cyano-ethylene	I_2
Metal-free Phthalocyanine (4.75)	Dark Conductivity	2×10^{-8}	2×10^{5}	10^{3}	5×10^{4}
	Photoconductivity	10^{-9}	8×10^{3}	4×10^{2}	3×10^{4}
	% Unpaired Spin	--	0.3(-)	0.5(-)	0.3(-)
Violanthrene (4.90)	Dark Conductivity	10^{-9}	9×10^{2}	--	--
	Photoconductivity	4×10^{-10}	1.5×10^{2}		
	% Unpaired Spin	--	100(+)		
Tetracene (5.05)	Dark Conductivity	2×10^{-9}	5×10	--	--
	Photoconductivity	3×10^{-10}	2×10^{2}		
	% Unpaired Spin	--	--		
Perylene (5.02)	Dark Conductivity	2×10^{-9}	4×10^{2}	--	--
	Photoconductivity	6×10^{-10}	10^{3}		
	% Unpaired Spin	--	0.1		

Electrical Properties of Organic Solids. Part IV.

THERMOELECTRIC POWER

David R. Kearns

The thermoelectric power measurement is a method used to determine the sign of the majority charge carrier in a semiconductor. In this measurement, one side of a sample is heated while the other side is cooled. Because of the temperature gradient, ΔT, the more mobile carriers diffuse away from the hot side of the sample, generating a small voltage $\Delta E = q\Delta T$ across the sample. From the sign of the voltage developed, one may determine the sign of the majority carrier. Although originally used with inorganic semiconductors, this method has also been applied to aromatic organic solids in an attempt to determine the sign of the majority charge carriers.[1]

In a previous paper,[2] the nature of the conducting species in a sample of metal-free phthalocyanine doped with small amounts of ortho-chloranil was discussed, and the conclusion reached was that holes (positive charges) were the majority carriers. However, in a system composed largely of ortho-chloranil with small amounts of metal-free phthalocyanine, experiments suggested that electrons carried the bulk of the current. In an attempt to further establish these two points, thermoelectric measurements were carried out on these two systems in the apparatus shown in Fig. 26. The two samples used for this measurement were prepared by grinding mixtures of phthalocyanine and ortho-chloranil in the desired proportions and then pressing them into a 1-cm-diameter disc, 0.2 cm thick. The sample that was primarily phthalocyanine contained 35 mole-% ortho-chloranil and the ortho-chloranil sample contained 2.5 mole-% phthalocyanine. The effect of the electrode-sample contact on the thermoelectric power measurements was studied for both samples by using various combinations of copper and aluminum electrodes. The results of these experiments are presented in Table VI. From these data it is clear that the voltage developed across the cell in this type of measurement is dependent upon the nature of the metal—organic sample contact. It appears that there is a large temperature-dependent contact potential between the metal and the organic sample. The voltage developed across the cell is thus a sum of two contact potentials and the voltage developed across the sample as a result of the temperature gradient. The data presented here indicate that unless the effect of contact potentials can be eliminated, it is not possible to determine with certainty the sign of the charge carriers in organic substances by a thermoelectric power measurement. The effect of other metal electrodes on thermoelectric power measurements is currently being investigated.

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

[2] D. R. Kearns, G. Tollin, and M. Calvin, J. Chem. Phys. 32, 1020 (1960); also David R. Kearns, Electrical Properties of Organic Solids. I. Kinetics and Mechanism of Photoconductivity of Metal-Free Phthalocyanine. II. Effects of Added Electron Acceptors and Donors (Thesis), UCRL-9120, March 1960 .

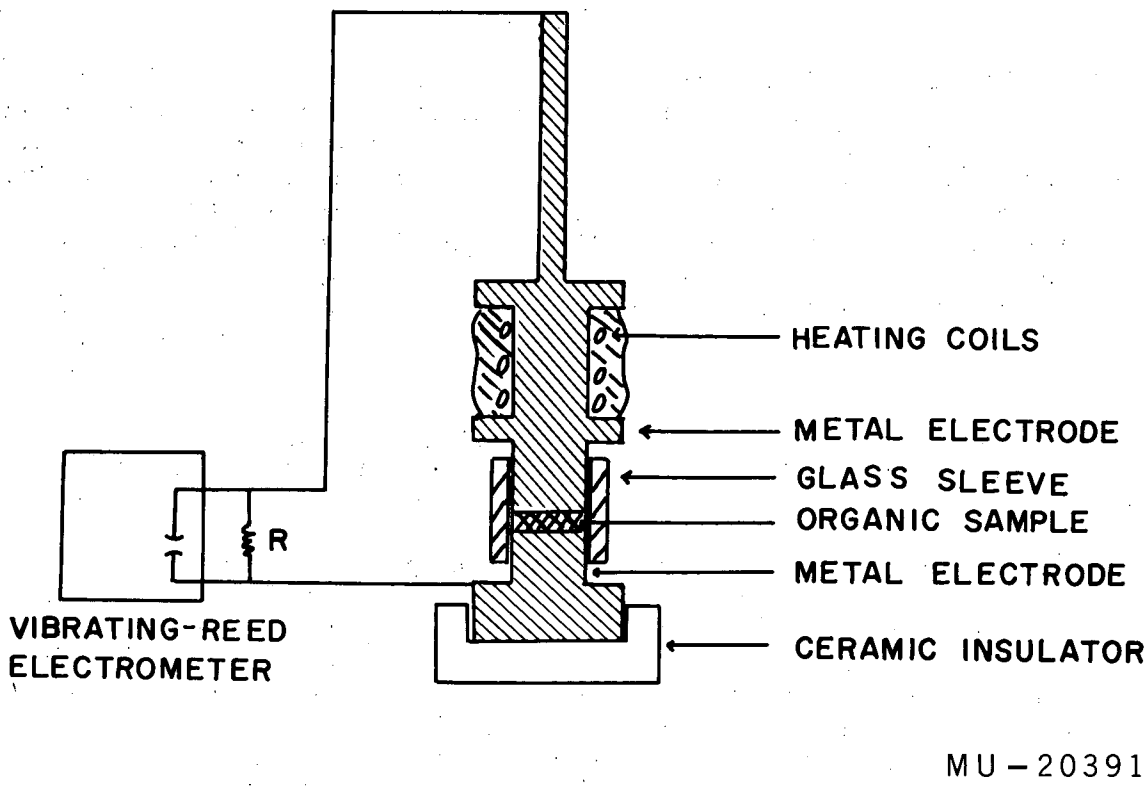

Fig. 26. Apparatus for thermoelectric measurements.

Cell Arrangement Hot Electrode Cold Electrode	Organic Material	Sign of Voltage Developed at Cold Electrode
Cu / organic / Cu	Ortho-chloranil + 2 1/2 mole % Phthalocyanine	+
Cu / Al / organic / Al / Cu	" "	+
Cu / organic / Cu	Phthalocyanine + 35 mole % Ortho-chloranil	+
Cu / Al / organic / Cu	" "	—
Cu / Al / organic / Al / Cu	" "	—

MU-20392

Electrical Properties of Organic Solids. Part V.

NON-OHMIC BEHAVIOR OF METAL-FREE PHTHALOCYANINE

David R. Kearns

An experiment result generally observed with aromatic organic compounds is that both the dark conductivity and photoconductivity cease to be ohmic at rather low strengths of electric field.[1, 2, 3] This usually occurs at field strengths around 10^3 v/cm; above this value the photo- and dark currents increase at a greater than linear rate with increasing voltage. At present there is no adequate explanation of this effect in organic solids.

As was discussed elsewhere,[4, 5] the current-voltage relationship

$$I \propto V^{3/2} \qquad (1)$$

was observed for surface cells of metal-free phthalocyanine.

These measurements, which were carried out only over a rather limited range of voltage, have now been extended to cover a much wider range. The techniques used in sample-cell preparation and in the electrical measurements are described elsewhere in detail.[4] The results of the extended current-voltage measurements are presented in Fig. 27, where the log of the current is plotted as a function of the log of the voltage for both the dark and photoconductivity of pure metal-free phthalocyanine (curves A and B). From this figure it is evident that both the dark current and the photocurrent are ohmic only up to a field strength of about 500 v/cm. Above this value both increase almost exponentially with voltage. A strikingly similar current-voltage curve has been obtained by Smith and Rose,[6] using relatively perfect insulating crystals of CdS having ohmic contacts. The explanation of the current-voltage curve given by Rose[7] is briefly as follows:

If an insulator of dielectric constant k is placed between two plane parallel electrodes separated by d cm, the capacitance C between the electrodes is

$$C = k/4\pi d \times 10^{-12} \text{ coulombs/volt.} \qquad (2)$$

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[1] D. D. Eley, Research Applied in Industry, 12, 293 (1959).

[2] A. T. Vartanian and I. A. Karpovich, Doklady Akad. Nauk (USSR), 3, 561 (1956).

[3] D. M. J. Compton, W. G. Schneider, and T. C. Waddington, J. Chem. Phys. 27, 160 (1957).

[4] David R. Kearns, Electrical Properties of Organic Solids. I. Kinetics and Mechanism of Photoconductivity of Metal-Free Phthalocyanine. II. Effects of Added Electron Acceptors and Donors (Thesis), UCRL-9120, March 1960.

[5] G. Tollin, D. R. Kearns, and M. Calvin, J. Chem. Phys. 32, 1013 (1960).

[6] R. W. Smith and A. Rose, Phys. Rev. 97, 1531 (1955).

[7] A. Rose, Phys. Rev. 97, 1938 (1955).

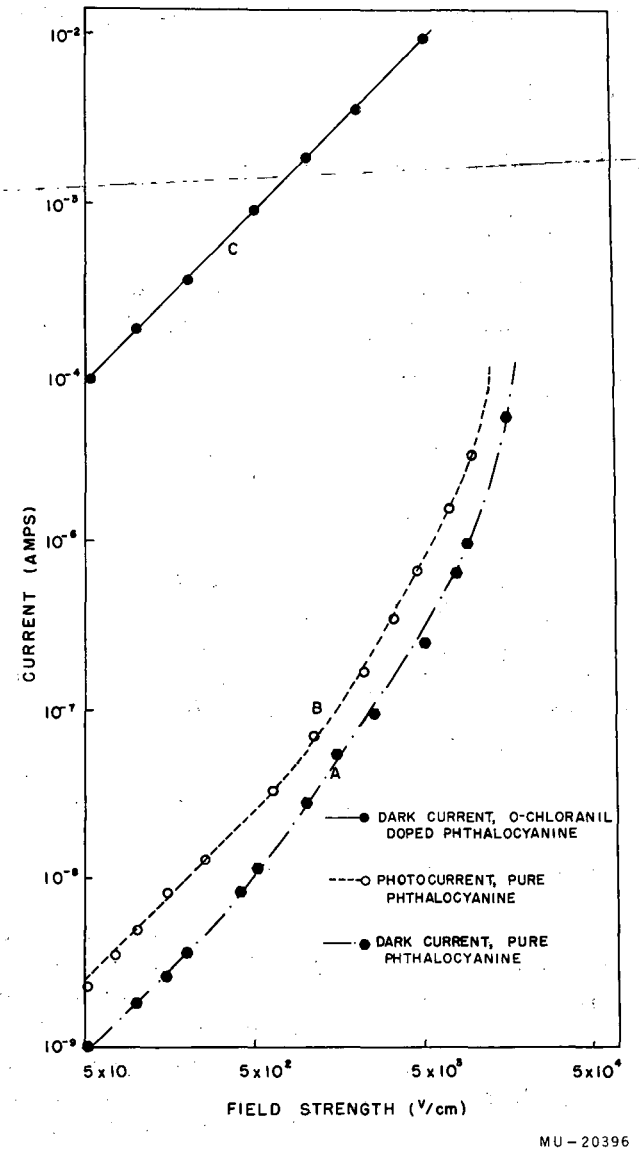

Fig. 27. Log plot of voltage for dark and photoconductivity of pure metal-free phthalocyanine.

The charge Q that can be accommodated in the space between the electrodes is

$$Q = CV. \tag{3}$$

The space charge-limited-current is then

$$I = Q/T, \tag{4}$$

where T is the transit time between the electrodes. For a charge drift mobility of μ, and an applied electric field E, the transit time is

$$T = d/E\mu = d^2/V\mu \ . \tag{5}$$

From this we find the space-charge-limited current is

$$I = 10^{-13} (V^2\mu k/d^3) \text{ amperes/cm}^2 . \tag{6}$$

This equation is valid only if there are no traps present with depths greater than approximately kT. If traps are present, however, only a fraction of the total charge forced into the insulator is free to donduct and consequently we must insert for the drift mobility the product of the drift mobility for free carriers and the fraction θ of the total space charge that is free. Equation (6) now becomes

$$I = 10^{-13} \ V^2(\mu_0 \theta)k/d^3 \text{ amperes/cm}^2 , \tag{7}$$

where μ_0 is the drift mobility of free carriers.

This same analysis may be now used to explain the current-voltage characteristics of pure metal-free phthalocyanine (curves A and B of Fig. 27). At sufficiently low voltages $V^2 \theta$ is small compared with V, and we should therefore expect a linear dependence of current upon voltage. In this region the carriers generated in the sample, either by light or thermally, outnumber those forced into the sample by the applied field. As the voltage on the sample is increased, there is a transition from ohmic current to space-charge-limited current behavior. Owing to the presence of traps, however, the space-charge-limited currents are reduced by a factor of θ from the value given in Eq. (6). Additionally, the space-charge-limited current does not vary with the square of the voltage at lower fields, since θ is a function of voltage in this region. This is because as voltage is increased more space charge is forced into the sample, a greater percentage of the traps becomes filled, and therefore a greater percentage of the injected charge is free to donduct. The net result is that the current increases with some power of the voltage greater than 2 until, at some very high voltage, all traps become filled ($\theta \longrightarrow 1$) and the space-charge-limited-current obeys Eq. (6). Apparently, the phthalocyanine current measurements have not been carried out at voltages large enough to fill all the traps. At such voltages one would not expect to observe photoconductivity.

The above analysis of the phthalocyanine current-voltage characteristics in terms of a combination of carriers generated in the phthalocyanine layer and carriers forced into the phthalocyanine from the electrodes is qualitatively able to explain the experimental data. If this analysis is correct, it follows that graphite electrodes form ohmic contacts with the phthalocyanine, a point of great interest.

The above analysis can be somewhat verified by the results of the following experiment. It was found previously[4, 8] that the addition of ortho-chloranil to a surface cell of metal-free phthalocyanine increases (by a factor of 10^5) both the dark and the photoconductivity of the phthalocyanine layer. It was concluded that the effect of ortho-chloranil was to remove electrons from phthalocyanine, thus greatly increasing the number of charge carriers (positive holes) and therefore the conductivity of the phthalocyanine layer. From the data presented in Fig. 27, an increase by a factor of 100 in the phthalocyanine conductivity would be large enough so that the current arising from bulk-generated carriers would be larger than the space-charge current. The addition of 5×10^{-6} mole of ortho-chloranil to a phthalocyanine surface cell increases the dark conductivity by a factor of 10^4. From such a sample one would expect ohmic behavior over the entire voltage range studied. That this was in fact observed is shown in Fig. 27, curve C, where the current-voltage curve for an ortho-chloranil "doped" phthalocyanine surface cell is presented. This supports the notion that the non-ohmic behavior of "pure" metal-free phthalocyanine is a result of a space-charge-limited current. The effect of this conclusion on the interpretation of the kinetics of photoconductivity in metal-free phthalocyanine is to be discussed elsewhere.

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[8] D. R. Kearns, G. Tollin, and M. Calvin, J. Chem. Phys. 32, 1020 (1960).

SOLID-STATE IONIZATION POTENTIALS OF SOME AROMATIC ORGANIC COMPOUNDS

David R. Kearns

Theories of the electrical properties of organic molecular crystals have often involved solid-state ionization potentials.[1,2] The gaseous ionization potential is an important property governing molecular behavior and, correspondingly, the solid-state ionization potential is an important parameter governing the behavior of molecules in the solid state. Because of our general interest in the electronic and electrical behavior of organic solids, the solid-state ionization potential is a parameter of great interest to us.

In 1957 Lyons calculated the energy required to ionize a molecular crystal in the ground state and compared this value with the energy required to ionize a molecule, from such a crystal, in the gaseous state.[1] Since there is polarization energy arising from the presence of a singly charged ion in a polarizable molecular crystal, less energy is required to ionize a molecule in a crystal than in the gaseous state. Using classical equations, Lyons estimated the polarization-energy contribution to the lowering of the ionization potential to be 0.8±0.3 ev for naphthalene and 1.0±0.3 ev for anthracene. One might also expect additional lowering of the crystal ionization potential due to resonance energy arising from possible migration, from molecule to molecule, of the positive charge produced in the crystal by ionization.

The gaseous ionization potential of anthracene has been estimated to be 7.2 ev,[3] and, consequently, a crystal ionization potential as low as 5.9 ev might be expected. This is barely within the quartz ultraviolet spectral region. Tetracene, on the other hand, has a gaseous ionization potential of approximately 6.5 ev,[4] and therefore should have a crystal ionization potential easily within the quartz uv energy region if Lyons's calculations are valid. It was consideration of these calculations that first suggested to us the feasibility of using quartz uv equipment to determine the solid-state ionization potential of organic compounds by means of the photoelectric effect.

Measurements involving the photoionization of organic solids were actually carried out as early as 1913 by Pauli,[5] who studied the photoionization of anthracene, β-naphthylamine, and eosin. Subsequent workers have studied the photoionization of other compounds (rosaniline[6] and cyanine dyes[7]); however, it appears that no effort has been made to determine the threshold of photoionization.

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[1] L. E. Lyons, J. Chem. Soc. 1957, 5001.

[2] G. G. B. Garrett, in Semiconductors, N. B. Hannay, Ed. (Reinhold Publishing Corporation, New York, 1959), p. 634.

[3] R. M. Hedges and F. A. Matsen, J. Chem. Phys. 28, 950 (1958).

[4] I. Bergman, Trans. Faraday Soc. 50, 829 (1954).

[5] W. Pauli, Ann. d. Phys., 40, 677 (1913).

[6] A. Predwoditelew and N. Netchaewa, Zeit. f. Phys. 29, 332 (1924).

[7] R. Fleischmann, Ann. d. Phys., 5, 73 (1930).

In the experiments described below, the light from a Bausch and Lomb hydrogen arc illuminator was passed through a Bausch and Lomb 500-mm grating monochromator and then focused onto the sample in a vacuum chamber. A diagram of the experimental apparatus is shown in Fig. 28. Electrons ejected from the sample are collected by a platinum screen, which is held at +90 volts with respect to the sample. A vibrating-reed electrometer was used to measure the current flowing through a 5×10^{11}-ohm resistor R, which was placed in series with the sample and battery.

The organic compounds chosen for these initial experiments were

metal-free phthalocyanine,
violanthrene,
decacyclene,
tetracene,
perylene,
phenothiazine.

Metal-free phthalocyanine was of particular interest to us because a number of other solid-state measurements had been performed on it that suggested it had a relatively low ionization potential[8] (i.e., low with respect to anthracene and tetracene). We had recently studied the electrical properties of violanthrene and were interested in the ionization potential of this compound. Tetracene, perylene, and decacyclene were included in this study because we already have some knowledge of their ionization potentials and have studied the electrical properties of tetracene and perylene. Phenothiazine was expected, on the basis of its chemical behavior, to have a very low ionization potential; it was therefore included to check this supposition.

These compounds were first purified by vacuum sublimation in a muffle furnace.[8] The purified material was then sublimed onto a glass slide to which a ring of graphite had been applied. A diagram of a sample cell prepared in this manner is shown in Fig. 29. The sample was then mounted in the vacuum chamber and light from the monochromator was focused at the center of the circle formed by the graphite electrode. In this manner no excitation was allowed to reach the graphite and a photoelectric effect from graphite was eliminated. Light that was not absorbed by the organic sample was absorbed by the supporting slide. Photoelectric emission of electrons from the glass was measured and found to be smaller by two orders of magnitude than that from the organic solids with a threshold at 2300 A.

During the measurements the monochromator wave-length drive was turned at a rate of 11 A/min, and a slit width of 0.5 mm (corresponding to approximately 17.5 A) was used. The spectral response of the relative number of quanta per sec from the monochromator is given in Fig. 30. These data were used to convert the measured photoelectric currents to current that would be observed for an equal rate of input of quanta at all wave lengths. In Fig. 31 the measured photoelectric current as a function of wave length is given for a tetracene sample. Qualitatively, the same

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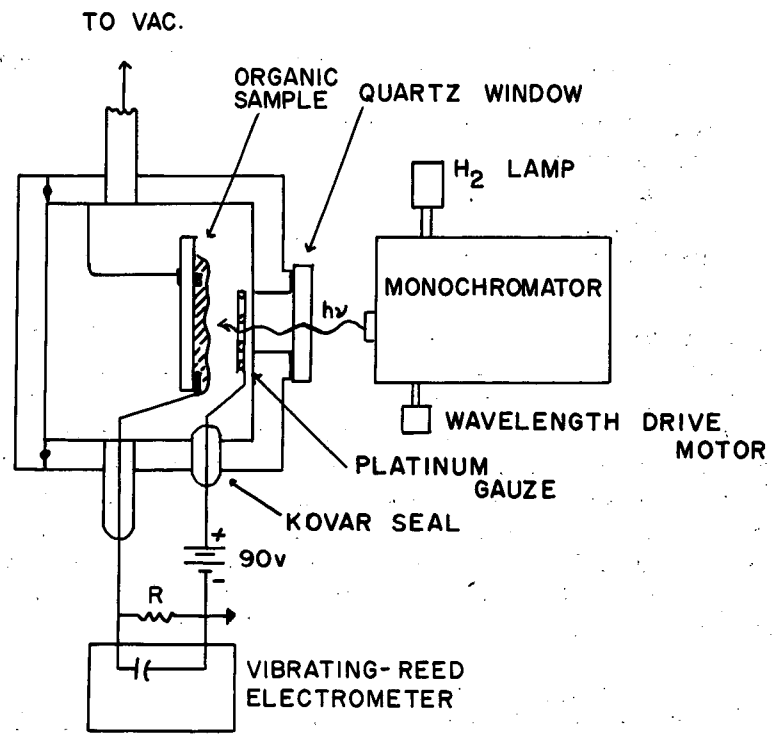
[8] D. R. Kearns, G. Tollin, and M. Calvin, J. Chem. Phys. 32, 1020 (1960).


TO VAC.
ORGANIC SAMPLE
QUARTZ WINDOW
H_2 LAMP
MONOCHROMATOR
hν
WAVELENGTH DRIVE MOTOR
PLATINUM GAUZE
KOVAR SEAL
+
90v
-
R
VIBRATING-REED ELECTROMETER

MU-20386

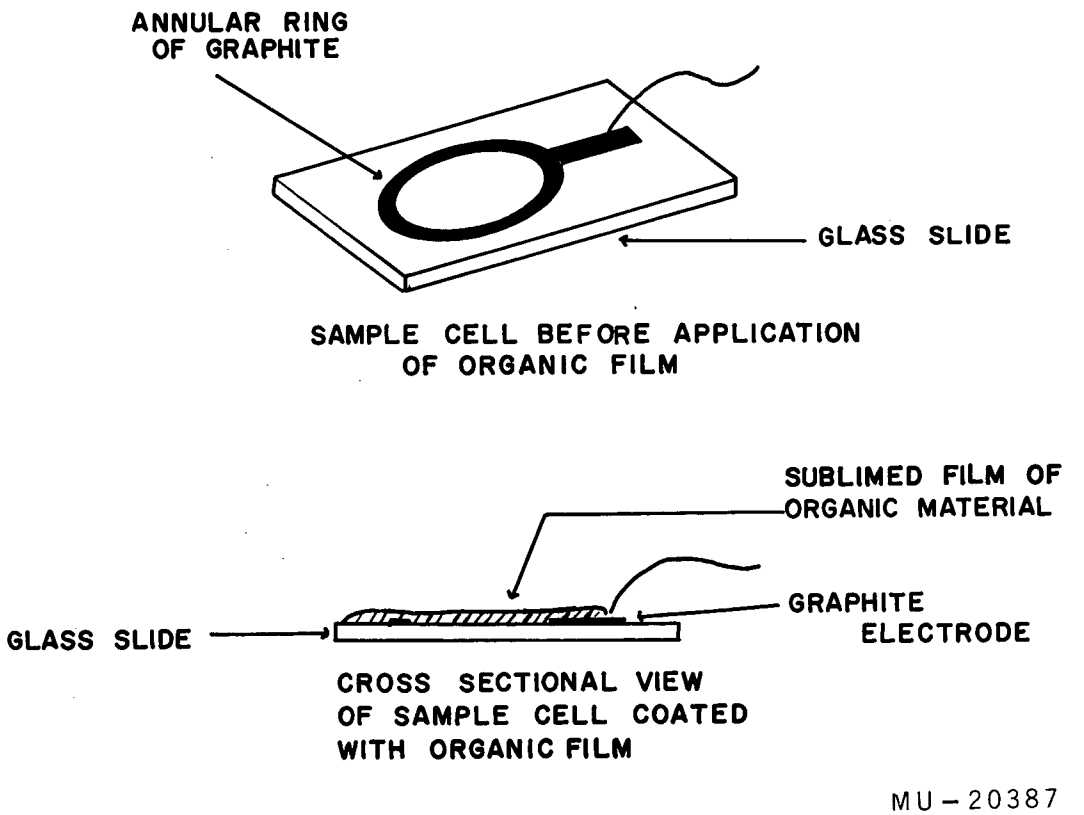
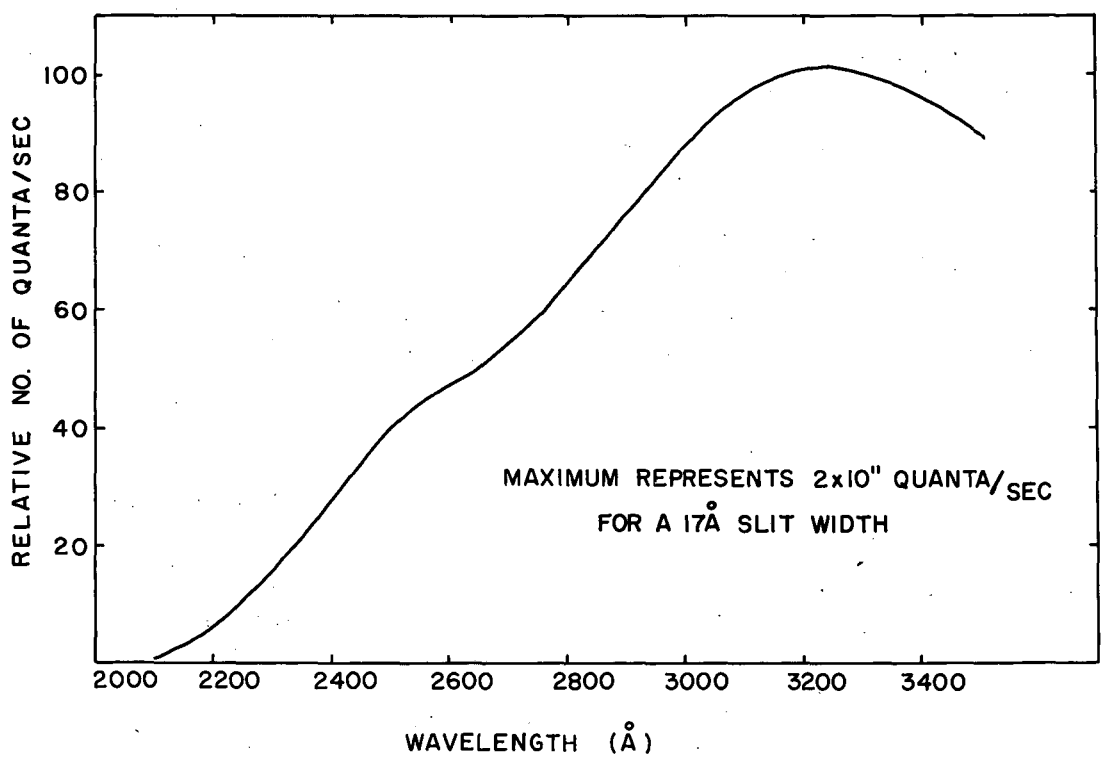


Fig. 29. Diagram of sample cell.



MU-20388

Fig. 30. Spectral response of quanta/sec from monochromator.

curves were obtained for the other compounds studied. It is difficult to determine from this type of plot the threshold wave length for photoionization. Quite likely the initial rise in photoelectric current with decreasing wave length is due to trace impurities with lower ionization potentials than the host material. This is suggested by the fact that with tetracene, for example, the long-wave-length threshold was shifted several hundred A to the red when impure material was used.

When a semilog plot of photoelectric current as a function of wave length is made, the current can be extrapolated in a straight line toward zero current. In Fig. 32 the log of the photoelectric current is given as a function of wave length for the six compounds studied. One now has the difficulty of deciding what the threshold value of the photoelectric current should be. If we arbitrarily choose a value of 0.5% of the maximum current observed, this corresponds to a current value of about 10^5 electrons/sec. A variation in the choice of this threshold current by a factor of 2 in either direction causes only a 0.05-ev variation in the calculated ionization potentials. The solid-state ionization potentials that were obtained by using this criterion are presented in Table VII.

It is of interest to note that the difference between the solid-state and gaseous ionization potentials are reasonably close to Lyons's original estimate.

These measurements are now being extended to include a larger number of organic compounds.

Table VII

Gaseous and solid-state ionization potentials of some aromatic organic compounds

Compound	Gaseous ionization potential	Solid-state ionization potential	Δ
Phenothiazine		4.65±0.05 ev	
Metal-free phthalocyanine		4.75	
Violanthrene		4.90	
Perylene	6.82	5.02	1.8
Tetracene	6.63	5.05	1.6
Decacyclene	6.42	5.09	1.3

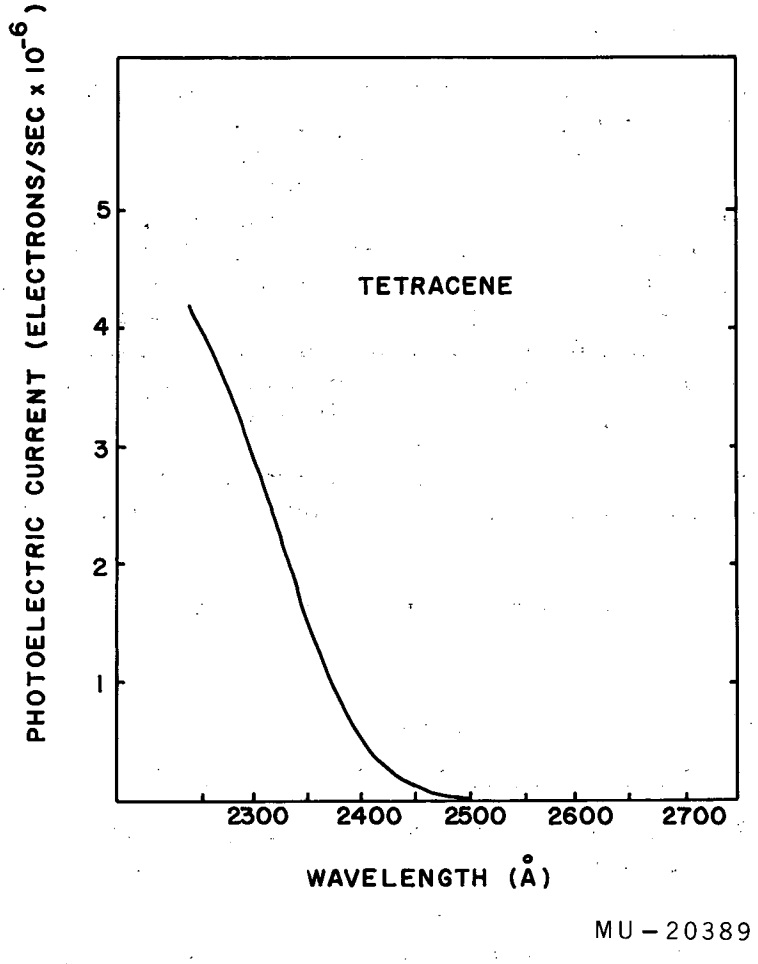


Fig. 31. Photoelectric current as a function of wave length, for tetracene.

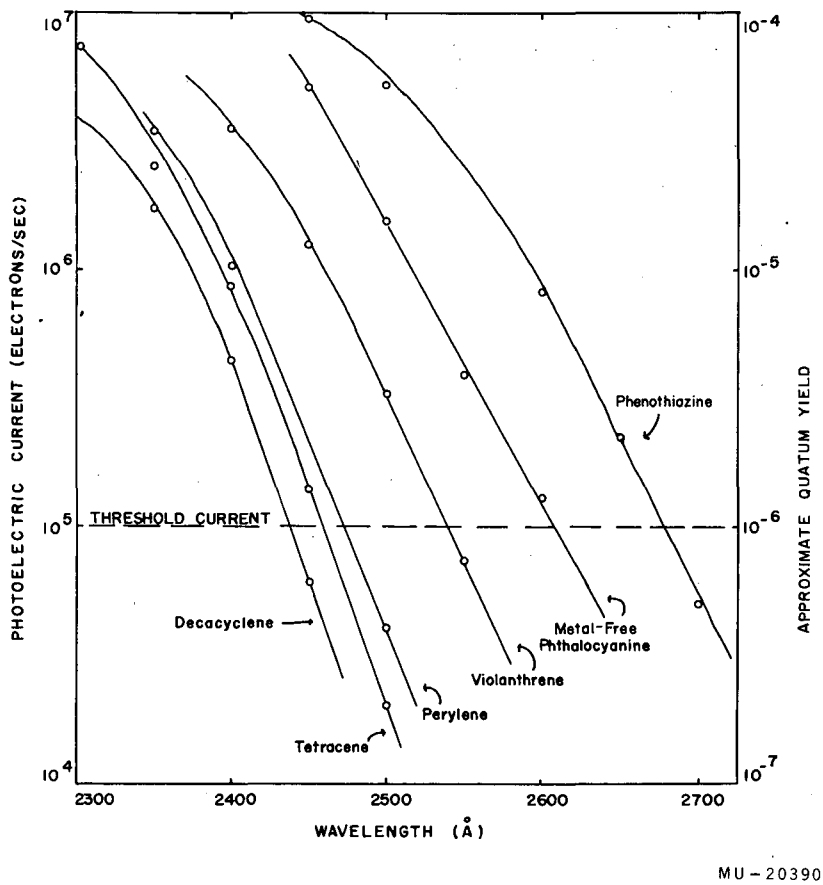


Fig. 32. Semilog plot of photoelectric current as a function of wave length.

BIODYNAMICS OF CARBON REDUCTION DURING PHOTOSYNTHESIS

James A. Bassham, Martha Kirk, and David Smith

In a previous report we described conditions under which it was possible to maintain steady-state photosynthesis of Chlorella pyrenoidosa in the presence of $C^{14}O_2$ for several hours.[1] A nutrient solution was used in which nitrogen is supplied in the form of ammonium ion. The nutrient solution is continuously and automatically renewed as the algae grow, and the algal density is maintained constant. Several experiments, during which these conditions were maintained, have now been completed. The sizes of total and "active pool" reservoir of amino acids, whose synthesis is of particular importance during photosynthetic carbon reduction, have been measured. The rates of flow of carbon through these amino acids have also been measured. These amino acids include alanine, aspartic acid, glutamic acid, serine, threonine, glutamine, and citrulline. The detailed results of these experiments, soon to be published in a separate report, are only summarized here:

1. There are at least two reservoirs of these amino acids, one of which is labeled very rapidly and one or more of which are labeled very slowly with C^{14} when the algae photosynthesize in $C^{14}O_2$.

2. Kinetic analysis shows that at least some of these amino acids (alanine, serine, aspartic acid, and probably glutamic acid) are formed directly from intermediates of the carbon-reduction cycle of photosynthesis without the intermediacy of free sugars. We believe these amino acids to be formed within, or in close association with, the chloroplasts. The "active pools" of these amino acids sometimes account for at least 70% of the externally measured NH_4^+ uptake, and 30% of the total CO_2 uptake.

3. When the light is turned off there is a short period (about 10 minutes) during which there is an accelerated uptake of NH_4^+. The total pools of these amino acids (see above) increase. A part of this "dark" synthesis uses unlabeled substrate; the specific radioactivity of the combined pool of each of these amino acids decreased.

4. Addition of unlabeled acetate to algae that have been photosynthesizing for 1 hour in $C^{14}O_2$ results in an increase in the total pools of glutamic acid, but the C^{14} content of this compound is not appreciably affected. Its over-all specific radioactivity therefore decreases. It may be that this synthesis of glutamic acid from acetate takes place in the "non-active" pool, that is, the one we believe to be outside the chloroplasts.

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

ROUTES TO THE CHEMICAL SYNTHESIS OF BRANCHED-CHAIN SIX-CARBON ACIDS

Emma J. McDonald

Introduction

Hamamelonic acid diphosphate, its epimer, and phosphates of other acids that appear to be closely related to hamamelonic acid have been found in the photosynthetic products of green algae.[1] It therefore is of interest to synthesize chemically the four isomeric 2-hydroxymethyl pentonic acids and to identify them and their lactones chromatographically, and to study the acid-lactose conversion. This investigation is concerned with the two branch-chained acids related to ribulose.

Hamameli tannin was isolated from the bark of the North American shrub Hamamelis virginiana (witch hazel) as a crystalline compound by Freudenberg[2] in 1919. Freudenberg and Blummel[3] showed that it had the composition of a digalloylhexose and that, on hydrolysis, two moles of gallic acid and one mole of a branched-chain hexose, hamamelose, were formed. These authors oxidized the hexose to hamamelonic acid from which they prepared a phenylhydrazide having a melting point of 202-203° and $[\alpha]^{22}_{578} = 35.2$ (aqueous acetic acid and pyridine). Schmidt[4] further identified the acid by reduction to methyl propyl acetic acid; he reported the structure of hamameli tannin as shown in Fig. 33. Hamamelonic acid was thus shown to be a 2-hydroxymethyl pentonic acid. Two epimeric pairs of this structure, in both the D and L series, are possible. Those of the D series are shown in Fig. 34. Schmidt and Heintz[5] reacted D- and L-ribulose and D- and L-xylulose with hydrogen cyanide and subsequently converted the cyanhydrins thus formed to the respective acids. Although isolation of the ketopentoses and separation of the epimeric product acids proved, in each case, to be difficult, the authors identified the phenylhydrazide of one of the acids prepared from D-ribulose with the phenylhydrazide of hamamelonic acid. Hamamelonic acid is therefore represented by I or II of Fig. 34. Since its phenylhydrazide has a positive optical rotation, it is presumed that it may be further identified as I by analogy with the behavior of gluconic acid as contrasted to that of mannonic acid.

In 1936 Levene and Tipson published "An Improved Method for the Preparation of Xylulose and Ribulose."[6] In their procedure, epimerization of xylose and of arabinose was carried out in hot pyridine solution, and the resulting sugars were converted to a mixture containing the respective isopropylidene derivatives. Thus, from arabinose they obtained di-isopropylidene-arabinose, diisopropylidene-ribulose, 1,5-anhydro-2,3-isopropylidene-ribose, and 2,3-isopropylidene-ribose. These mixtures can be fractionally distilled under reduced pressure. However, the boiling

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[1] V. Moses and M. Calvin, Proc. Natl. Acad. Sci. U.S. 3, 260 (1958).

[2] Freudenberg, Ber. deut. Chem. Ges. 52, 177 (1919).

[3] Freudenberg and Blummel, Ann. 440, 51 (1924).

[4] Schmidt, Ann. 476, 250 (1929).

[5] Schmidt and Heintz, Ann. 515, 77 (1935).

[6] Levene and Tipson, J. Biol. Chem. 115, 731 (1936).

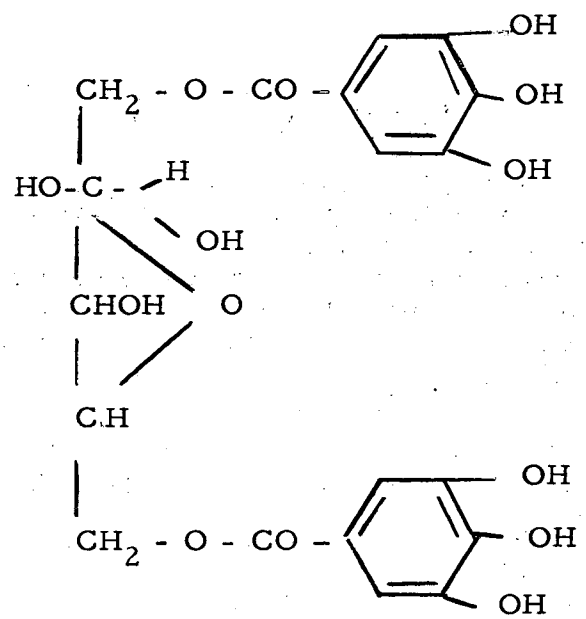
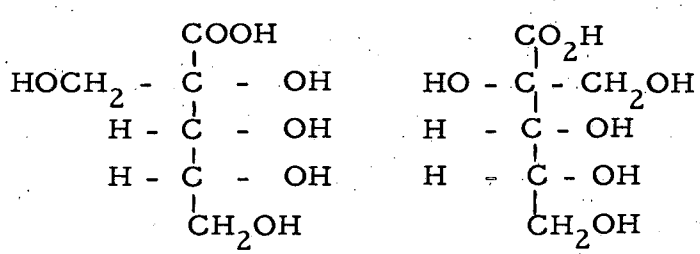


Fig. 33. Structure of hamameli tannin.



I II

Fig. 34. Epimeric forms of D-hamamelonic acid.

points of the diisopropylidene derivatives and that of the monoisopropylidene-anhydroribose are sufficiently close that complete separation cannot be accomplished.

We have found that diisopropylidene arabinose, diisopropylidene ribulose, and 2, 3-monoisopropylidene-1, 5-anhydro-ribose can be separated by means of gas chromatography. A chloroform solution of the sample is introduced into an Apiezon column maintained at 170° and through which helium is passed at the rate of 75 ml per minute. The characteristic (and reproducible) rate of flow of each compound through the column is sufficiently different that complete separation is accomplished. In Fig. 35 the curves from four chromatographs are superimposed so that the initial rise due to the chloroform is common to all. It includes (a) diisopropylidene arabinose, (b) 2, 3-isopropylidene-1, 5-anhydro-ribose, (c) a mixture of the above, and (d) a fraction of the reaction mixture.

This procedure provides a means of rapidly analyzing such mixtures and it also affords a method of separation and purification.

Purification of Diisopropylidene-D-Ribulose

Diisopropylidene-D-ribulose was separated from a mixture of isopropylidene derivatives (Fig. 36) boiling in the range of 60-70° (0.1 mm pressure), by means of an Apiezon "Megachrom" column. The separation was carried out in 2-g batches, and collections were made so as to accumulate the ribulose derivative in a cell containing glass wool. The diisopropylidene ribulose was subsequently removed from the wool by chloroform extraction. A gas chromatograph (Fig. 37) of the filtered chloroform extract indicates the completeness of separation. After removal of the chloroform by evaporation under somewhat reduced pressure, the remaining material was distilled (bath temperature 65°) at less than 0.05 mm pressure. Diisopropylidene thus prepared crystallized when cooled in an acetone-dry ice mixture; m. p. 25° (uncorr.), $[\alpha]_D^{25} = -110.1°$ (c = 3.88 g/100 ml acetone).

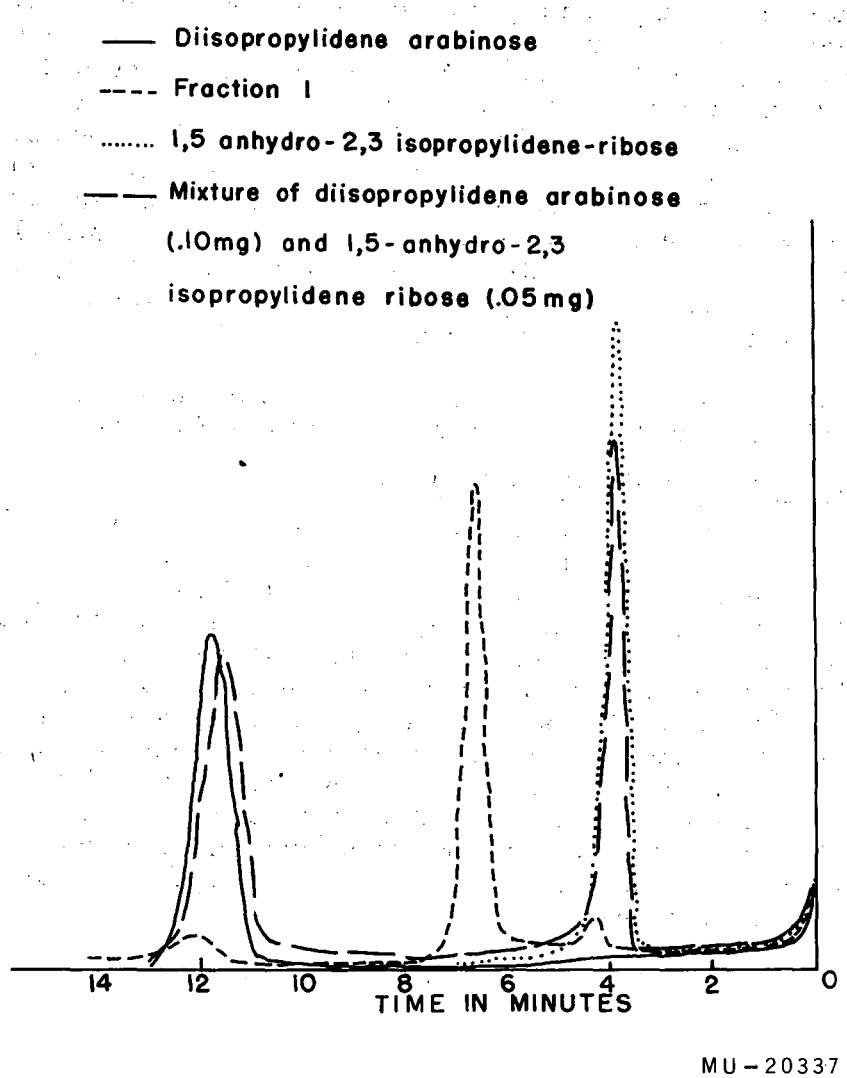


Fig. 35. Fraction of reaction mixture.

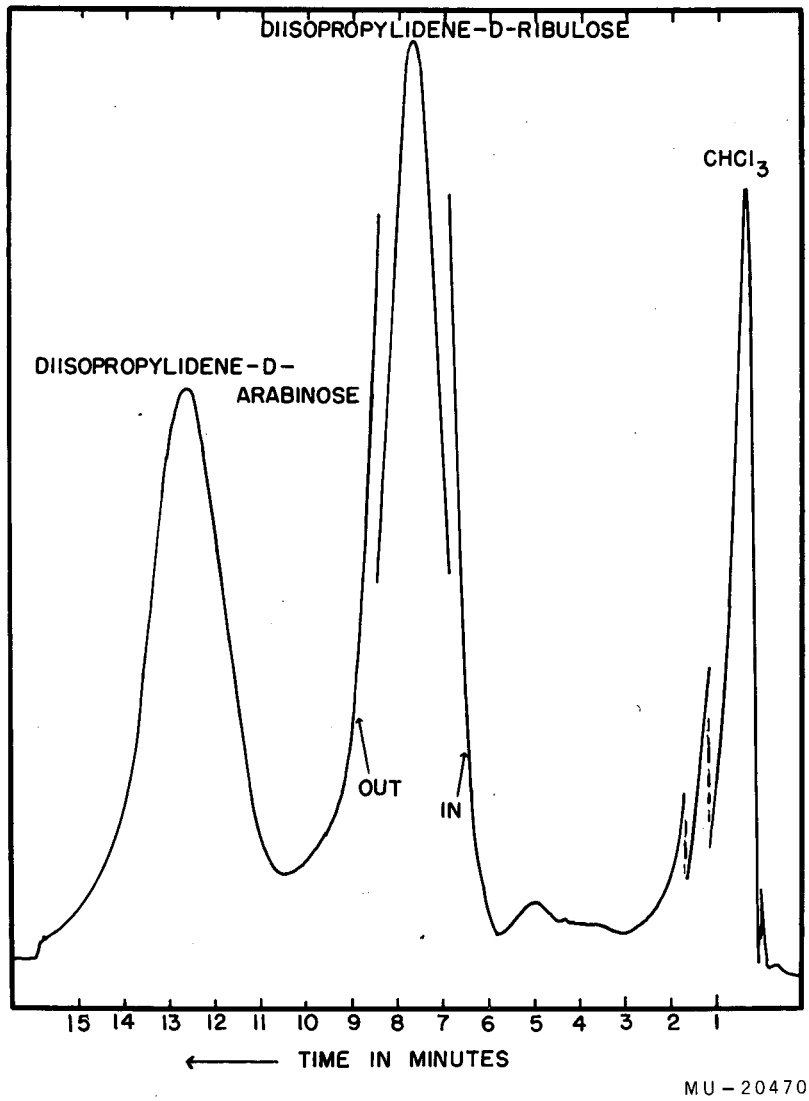


Fig. 36. Separation of diisopropylidene-D-ribulose from mixture of isopropylidene derivatives.

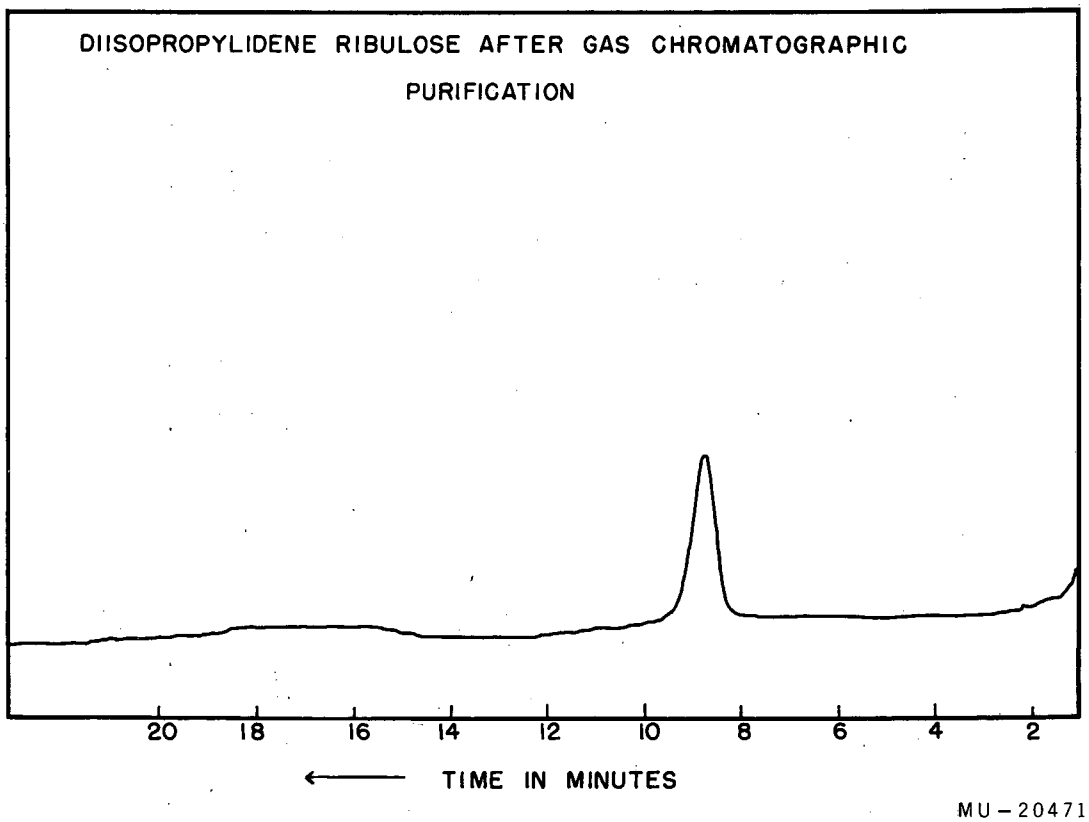


Fig. 37. Gas chromatograph of chloroform extract of diisopropylidene ribulose.

SEARCH FOR UNSTABLE FIXATION PRODUCT IN ALGAE BY METHYLATION

Zofia Kasprzyk

Lynen and coworkers found that β-methyl-crotonyl-CoA cocarboxylase contains biotin as the coenzyme.[1] The enzymatic activity was determined by the formation of radioactive β-methyl-glutaconyl-CoA from β-methyl-crotonyl-CoA, radioactive CO_2, and ATP in the presence of an enzyme prepared from Mycobacterium. The quantity of biotin, as determined by bacteriological test, increased during the purification of the enzyme proportionally with the increase of enzymic activity. In this reaction, an unstable CO_2-biotin-enzyme complex was presumed formed which then transferred its CO_2 to free biotin in the medium. This latter compound could then be stabilized by methylation. The carboxylation reaction was performed in a solution containing Tris buffer (pH 8.0), biotin, ATP, $MgSO_4$, $KHC^{14}O_3$, and purified cocarboxylase. The carboxybiotin formed under these conditions was very unstable, especially in acid solution. At pH 4.5, the compound lost all of its radioactivity in the form of CO_2 after 25 minutes at 0°. It was more stable in 0.033 N KOH, in which it lost 7% of the radioactivity after 30 minutes at 0°, and 22% at 20°. The authors suggested that the CO_2-biotin is a derivative of an allophanic acidlike structure of biotin with the carbomethoxy group attached to one of N atoms of biotin.

After lyophilization of the reaction mixture containing the unstable complex, the dried residue was suspended in 90% methanol and treated with an ethereal solution of diazomethane, giving the stable methyl ester of the CO_2-biotin complex. This compound can be extracted by ether and separated from biotin, and from the monomethyl ester of biotin, by paper chromatography using water-propanol (15:85 by volume) as the solvent. The R_f of methylated CO_2- biotin is 0.23, and of the methyl ester of biotin, 0.37. These experiments of Lynen and co-workers suggested the possibility that some unstable products of carboxylation in photosynthesis can be stabilized by methylation and that biotin may play a role in the primary carboxylation reaction. To investigate the first of these problems, the methylation--at low temperatures in alkaline solution--of photosynthesis products and the identification of methylated compounds were tried. To determine whether biotin is the acceptor of CO_2 in the carboxylation reaction in photosynthesis, the simplest method is to identify the radioactive synthetic methyl ester of CO_2-biotin with methylated products of photosynthesis in the presence of free biotin.

Lynen et al. synthesized the methyl ester of carbomethoxybiotin (m. p. 130-131°) by treating the methyl ester of biotin (m. p. 166°) with carbomethoxychloride in chloroform. This compound crystallized as colorless prisms that could be recrystallized from ethyl acetate and petroleum ether or water. Lynen found that when this compound was mixed with the radioactive compound obtained enzymatically the specific activity of the mixture remained unchanged even after several crystallizations. These results were taken as criteria for the identity of the two compounds.

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[1] F. Lynen, J. Knappe, E. Lorch, G. Jutting, and E. Ringelmann, Angew. Chem. 71, 481 (1959).

The carbomethoxychloride can be prepared by the action of methyl alcohol on phosgene:

$$COCl_2 + 2CH_3OH \longrightarrow ClCOOCH_3 + CH_3Cl + H_2O .$$

Phosgene is prepared by the action of carbon tetrachloride with fuming sulfuric acid according to the reaction

$$2H_2SO_4 + 3CCl_4 \longrightarrow 3COCl_2 + 4HCl + S_2O_5Cl_2 .$$

Using radioactive carbon tetrachloride for the preparation of carbomethoxychloride, one could obtain the carbomethoxybiotin labeled in the carboxyl group; starting with radioactive methanol, one could get the label in the methyl group.

The Methylation of the Products of Photosynthesis

The experiments were performed as usual in two small vessels, each containing 5 ml of a 2% suspension of Chlorella (100 mg wet cells). The algae were preilluminated for 20 minutes in a stream of 1.6% CO_2 in air, and allowed to photosynthesize with 250 λ of 0.026 N $NaHC^{14}O_3$ (0.4 mc/ml) for 1 min. They were then killed with 20 ml of methanol and poured quickly into a flask containing 0.5 ml of 1 M Tris buffer (pH 8.4). The mixture was evaporated to dryness in vacuum at a bath temperature of 0°. The residue was treated with 5 ml of methanol, and divided into two equal parts. To one of them was added 10 ml of an ethereal solution of diazomethane and to the other, 20 ml of ether. Both samples were left overnight in the cold room at 4°. The next day they were filtered, and the filtrate was concentrated at 0° to 4 ml. The radioactivity of each sample was determined by means of a Geiger-Mueller counter. In the methylated sample, after concentration to 4 ml, a precipitate was formed (but none was formed in the nonmethylated control). The precipitate was filtered and dissolved in ether acidified with acetic acid. The total radioactivity of the samples was

I. Unmethylated sample, 230,690 cpm;

IIa. Methylated filtrate, 150,880 cpm;

IIb. Methylated precipitate, 60,000 cpm .

The solutions were put on chromatograms and run for 3 hr in propanol: water (15:85) (neutral solvent) and 3 hr in butanol:methyl ethyl ketone: water: dimethylamine (20:20:10:2) (alkaline solution). The chromatograms were put on photographic films, and the films were developed after 6 days' exposure to the chromatograms.

The unmethylated sample did not move in the direction of the neutral solvent, and it streaked in the alkaline solvent. (With light loading of the paper, the alkaline solvent occasionally showed a separation into discrete spots.) Both the methylated filtrate IIa and the precipitate IIb moved in the neutral solvent, but the former, IIa, moved more slowly than the latter (IIb). Both streaked in the alkaline solvent. The radioactive spots were cut out from the chromatograms and eluted successively (a) with ether, (b) with methanol, and (c) with 20% aqueous ethanol and with water, which were later combined. The extracts were concentrated separately and their radioactivities determined. The water (c) and ether (a) extracts from the unmethylated samples contained approximately 10% of the radioactivity each, while 80% was in the methanol extract. In the extracts from methylated samples the radioactivity was distributed in the following way: In the spot from the filtrate, IIa: in ether (a) 2%, in methanol (b) 75%, and in water (c) 23%. In the water extract (c) from this sample a crystalline precipitate was formed, melting at 150-155°. In the spot from the methylated precipitate, IIb, the following quantities of radioactivity were found: in ether (a) 3%, in methanol (b) 91%, and in water (c) 6%.

The extracts from each sample were again, separately, put on paper and run 3 hr in the propanol:water solvent. The radioactive spots from the unmethylated sample that was extracted with ether and methanol did not move, while the one extracted with water moved with the solvent front.

The radioactive spots from the methylated samples (both filtrate IIa and precipitate IIb) extracted with methanol (b) and water (c), all moved near the solvent front, as did the ether (a) extract of the methylated precipitate IIb. The ether extract (a) of the methylated filtrate IIa, on the other hand, had a smaller R_f value in the propanol-water system. All radioactive fractions of the methylated filtrate IIa showed, after the second chromatography and on being treated with the Hanes-Isherwood phosphate spray, a positive test (blue color) after they had stood for 2 days at room temperature.

The Preparation of Phosgene

Phosgene was prepared by the procedure described by Vogel.[2]

The Preparation of Carbomethoxychloride

This reagent was prepared by the procedure for carbobenzoxychloride described by Bergmann and Zervas.[3] To a 20% solution of phosgene in toluene (previously cooled in an ice bath), methyl alcohol was added dropwise, with stirring, over a period of 1/2 hr. After the solution was stirred for an additional 2 hr at room temperature, the excess phosgene was removed by passing a stream of dry N_2 through the reaction mixture for 1 hr. The mixture was then poured into a separatory funnel and the lower layer of $ClCOOCH_3$ was separated from the upper (toluene) layer. The carbomethoxychloride layer was dried with Na_2SO_4 and stored in the refrigerator.

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[2] A. I. Vogel, Textbook of Practical Organic Chemistry, (Longmans, Green, New York, 1956), p. 185.

[3] M. Bergmann and L. Zervas, Ber. deut. Chem. Ges. 65, 1192 (1932).

The Preparation of the Methyl Ester of Biotin

Diazomethane was prepared by slowly adding crystals of N-methyl-N-nitroso-N'-nitroguanidine to a 50% aqueous solution of NaOH that was covered with ether and cooled in ice. When the reaction was terminated, the ethereal solution of diazomethane was distilled from the reaction mixture (water bath at 40°) into a receptacle containing a small quantity of ether that was cooled by an ice bath. The methylation of biotin was accomplished by reacting 1 g of biotin, dissolved in 30 ml of 90% methanol, with an excess of diazomethane in the ether solution. In general, the reaction was completed (i.e., all biotin crystals were dissolved) in 15 min; if not, more 90% methanol was added. The reaction mixture was left for a few hours in the cold room, and then overnight in the deep freeze (-15°). The precipitated crystals of monomethylbiotin were filtered off. The filtrate, after concentration at 0° in vacuum, gave a second crop of crystals. The monomethyl-ester of the biotin, melting at 166°, was formed in nearly quantitative yield.

The Preparation of the Methyl Ester of Carbomethoxybiotin

The monomethyl ester of biotin (100 mg) was dissolved in 1 ml of chloroform, mixed with 1 ml of dry $ClCOOCH_3$, and allowed to stand overnight in the refrigerator. The next day, just enough pyridine (about 1 ml) was added (at 0°) to neutralize the excess $ClCOOCH_3$; the mixture was then placed in the deep freeze (-15°). The methyl ester of carbomethoxybiotin crystallizes as colorless prisms. These were filtered quickly (cold) and dried in vacuum. The melting point after recrystallization from $CHCl_3$ was 130-131°.

EFFECT OF METHANOL ON THE DISTRIBUTION OF EARLY PRODUCTS OF $C^{14}O_2$ FIXATION

Zofia Kasprzyk

Experiments of Ouellet and Lefrançois[1, 2, 3] on the photosynthetic assimilation of $C^{14}O_2$ by Scenedesmus in the presence of different inhibitors have shown that it is possible to preserve nearly normal rates of assimilation into several compounds even when assimilation is almost entirely suppressed in other compounds.

Several compounds, even though they become labeled with C^{14}, are not considered as intermediates in the photosynthetic carbon reduction cycle. These are malic acid, aspartic acid, glycolic acid, serine, alanine, and glycine. Many inhibitors have been found that divert the fixation of radioactive carbon from the main path of carbon to some subsidiary compounds.[4, 5, 6]

Ouellet and Lefrançois, using the inhibitors that supress the main photosynthetic cycle, were looking for intermediates resistant to this inhibition and participating in an alternative pathway. These compounds may therefore play only a secondary role under normal conditions. Methanol, ethanol, acetone, o-phenanthroline, and ultraviolet radiation were used as the inhibitors. The authors found that 15% methanol, 12% ethanol, or 12% acetone totally inhibits the fixation of carbon. However, in the presence of methanol of concentrations up to 12%, ethanol up to 6%, or acetone up to 7% (by volume) the photosynthetic fixation of $C^{14}O_2$ into glycolic acid, glycine, alanine, serine, and an unknown compound proceeds at nearly normal rates over periods of 1 to 8 minutes; fixation into other compounds such as phosphoglyceric acid, phosphopyruvic acid, aspartic acid, and lipids is suppressed over periods of several minutes. The authors interpret these results by suggesting that the simple acids are intermediates in an alternative pathway, the initial step being the carboxylation of phosphoenolpyruvic acid.

The results of Ouellet and Lefrançois (Table VIII and Fig. 38) show that their control experiments did not achieve the same labeling patterns of different compounds as were found by Benson et al.[7] After 8 minutes the most highly labeled compounds (containing more than 50% of the total radioactivity) were glycolic acid and alanine. Phosphoglyceric acid and

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[1] M. Lefrançois, Recherches sur la photosynthèse (Thèse) Université Laval, Quebec, 1957.

[2] M. Lefrançois, and C. Ouellet, Can. J. Botany 36, 457 (1958).

[3] M. Lefrançois, and C. Ouellet, Can. J. Botany 37, 499 (1959).

[4] J. A. Bassham, A. A. Benson, and M. Calvin, J. Biol. Chem. 185, 781 (1950).

[5] R. W. Newburgh and R. H. Burris, Arch. Biochem. Biophys. 49, 98 (1959).

[6] C. Ouellet, J. Exptl. Botany 2, 316 (1951).

[7] A. A. Benson, S. Kawaguchi, P. M. Hayes, and M. Calvin, J. Am. Chem. Soc. 79, 4977 (1952).

malic acid contained very small quantities of radioactivity, in contrast to Benson's finding that these are generally the most radioactive over the same period of exposure to $C^{14}O_2$. These discrepant results may be due to the conditions under which Ouellet and Lefrançois performed their experiments. They preilluminated algae for 10 minutes in the absence of CO_2 and O_2. Afterwards, the algae (0.2 g of wet cells in 30 ml of suspension) were allowed to undergo photosynthesis in the presence of $Na_2C^{14}O_3$ (50 λ containing 5 μC, concentration unspecified).

In 1951 Calvin et al.[8] observed that if plants are illuminated in $C^{14}O_2$ until labeled C_3 and C_4 compounds appear, and then illumination is continued in the absence of CO_2, the labeled C_3 and C_4 compounds disappear and labeled glycolic acid and glycine on radioautograms are concomitantly increased. The small quantity of radioactive CO_2 that is given after the 10-minute period of illumination of algae in the absence of CO_2 could not change, in the experiment of Ouellet and Lefrançois, the distribution of carbon already established during this 10-minute period. Hence the flow of tracer remains shifted in the direction of the formation of glycolic acid and simple amino acids. These compounds (glutamic acid, alanine, serine, glycine, malic acid, glycolic acid, and the unknown compound) are also most resistant to the influence of inhibitors. They persist even in the presence of 15% methanol.

The purpose of the work reported here was to examine the influence of methanol on the incorporation of radioactivity in different compounds under normal photosynthetic conditions and to attempt the identification of the unknown compound.

Experimental Procedures

The experiments were performed as usual in small vessels containing 5 ml of a 2% suspension in 0.001 M ammonium phosphate of Scendesmus (100 mg of wet cells in each vessel). The algae were illuminated 15 minutes before the experiment in a stream of approx 2% CO_2 in air or other gas (H_2 or N_2). Afterwards, to half of the vessels 300 λ of methanol was added to make the methanol 6% by volume. In some experiments 10% methanol was used. After an additional 4-min illumination, 250 λ of 0.0268 N $NaHC^{14}O_3$, containing 100 μC, was added for times ranging from 5 sec to 8 min. The control experiments were performed in the same way, but without methanol. The algae were killed with 20 ml of methanol and extracted successively with boiling acetone, 80% ethanol, 20% ethanol, and water. The combined extracts were concentrated to small volumes, and different quantities of extracts corresponding to 100,000 cpm were put on paper chromatograms and run for 12 hr in phenol-water and 8 hr in butanol—propionic acid—water. The radioactivity of the suspensions and the extracts of algae was counted, either as residues on aluminum plates or as spots on paper chromatograms, by means of a G-M counter.

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[8] M. Calvin, J. A. Bassham, A. A. Benson, V. H. Lynch, C. Ouellet, L. Schou, W. Stepka, and N. E. Tolbert, Symposia Soc. Exptl. Biol. 5, 284 (1951).

Table VIII

Photosynthetic fixation in control experiment by Ouellet and Lefrançois
(cpm for 10 mg of algae)

	Duration of illumination (min)							
Compound	0.5	0.5	1	1.5	2	3	5	8
PGA	30	62	95	99	129	165	169	277
PEP	21	8	64	104	119	101	187	178
Aspartic acid	--	--	35	41	77	101	82	130
Glutamic acid	--	--	--	--	--	20	31	86
Alanine	39	42	245	424	520	666	1076	1176
Serine	9	3	74	74	277	403	478	496
Glycine	12	8	71	71	156	183	200	207
Malic acid	40	47	143	143	238	246	260	277
Glycolic acid	32	31	266	266	864	1101	2604	4225
Fumaric acid	--	--	20	20	47	51	76	76
Glyceric acid	--	--	--	--	--	--	--	30
Saccharose	--	--	--	--	51	58	80	148
Lipids	--	--	--	--	--	34	41	176
Unknown	128	82	316	418	440	552	539	419
Total	311	283	1359	1660	2918	3681	5873	7801

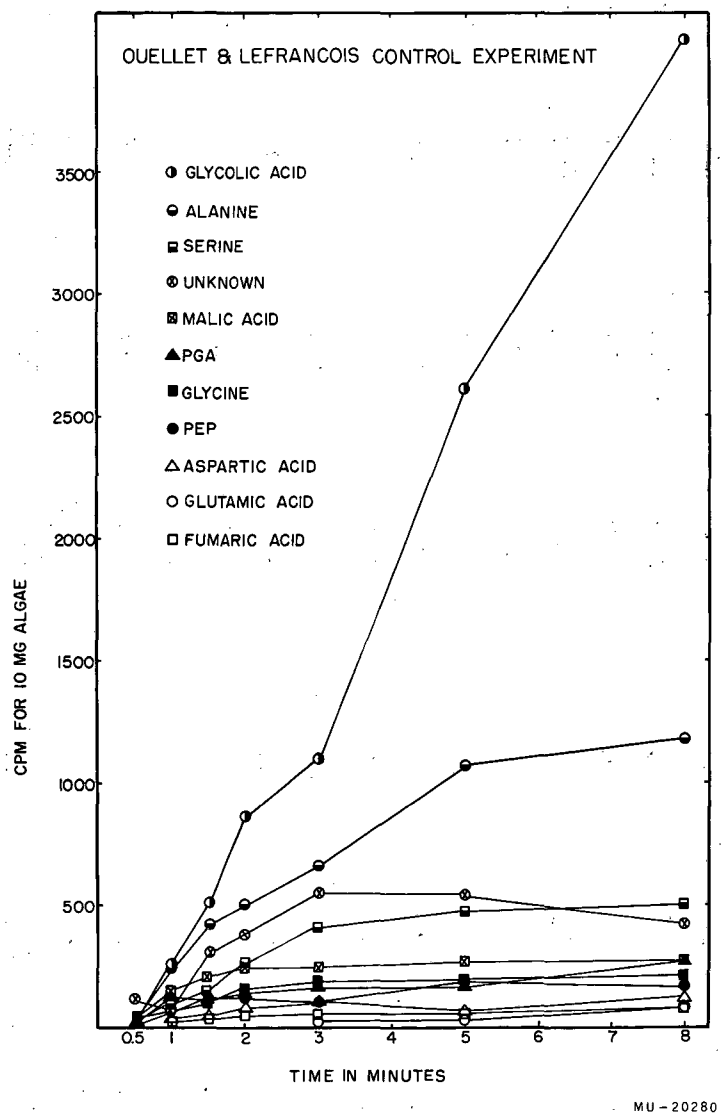

Fig. 38. Photosynthetic fixation in control experiment by Ouellet and Lefrançois (cpm for 10 mg of algae).

The identification of uridine diphosphoglucose (UDPG) was performed by chromatography of the eluted radioactive spot with carrier UDPG, and then spraying the paper with Hanes-Isherwood spray. Further confirmation was achieved by phosphatasing the spot with Polidase-S and then cochromatographing the product with carrier glucose.

Results

The results are represented in Tables IX through XIV and in Figs. 39 through 44. In the tables is presented the radioactivity (in cpm calculated for 10 mg of algae) fixed in different compounds in the absence and in the presence of methanol.

Tables IX and X give the results for CO_2 in air, Tables XI and XII for CO_2 in nitrogen, and Tables XIII and XIV for CO_2 in hydrogen. Figure 39 shows the total fixation of $C^{14}O_2$. One can see that in the absence of methanol the total fixation is practically the same in air, nitrogen, and hydrogen. The presence of 6% methanol causes a depression in all cases, the least in air (approx. 25%), more in hydrogen (approx. 40%), and the greatest in nitrogen (approx. 50%). Ten percent methanol in air caused a 50% depression of the total fixation.

Figure 40 shows the changes in fixation in monophosphate (MP), disphosphates (DP), phosphoglyceric acid (PGA), and malic acid. The fixation in monophosphates (Fig. 40) and malic acid (Fig. 41) follows the same scheme. The depression (in MP and malic acid), in the presence of methanol in air is not very great. In nitrogen, the fixation both with and without methanol is less than in air. In hydrogen one observes a very great (and reversible) fixation (much larger than in air) in the absence of methanol, and a very small one in its presence. In the diphosphates (Fig. 42) the fixation is not much affected by the atmosphere but the depression by the methanol is greater in H_2 and N_2 than in air.

In phosphoglyceric acid (Fig. 43) one sees, at the beginning of the photosynthesis, greater fixation in nitrogen and air than in H_2. The fixation in hydrogen is very small, either in the presence or absence of methanol. The methanol depression is greatest in N_2.

In Fig. 44 the fixation in UDPG is shown. It is the greatest in air, the depression caused by methanol being small. The fixation in nitrogen is smaller than in hydrogen (in shorter times); in both it is depressed more by the methanol than in air.

In Figs. 45, 46, 47, 48, and 49 is shown the fixation into the following amino acids: alanine (Fig. 45), aspartic acid and glycine (Figs. 46 and 47), glutamic acid (Fig. 48), and serine (Fig. 49). The fixation into alanine is very much influenced by the atmosphere. It is greatest in hydrogen, less in air, and least in nitrogen. In all cases, at the beginning of photosynthesis one observes greater fixation in the absence of methanol, but after 45 seconds in air, 2 min 30 sec in hydrogen, and 5 min 40 sec in nitrogen, the fixation begins to be much greater in the presence of methanol. In glycine the fixation is always less in the presence of methanol, being the greatest in hydrogen

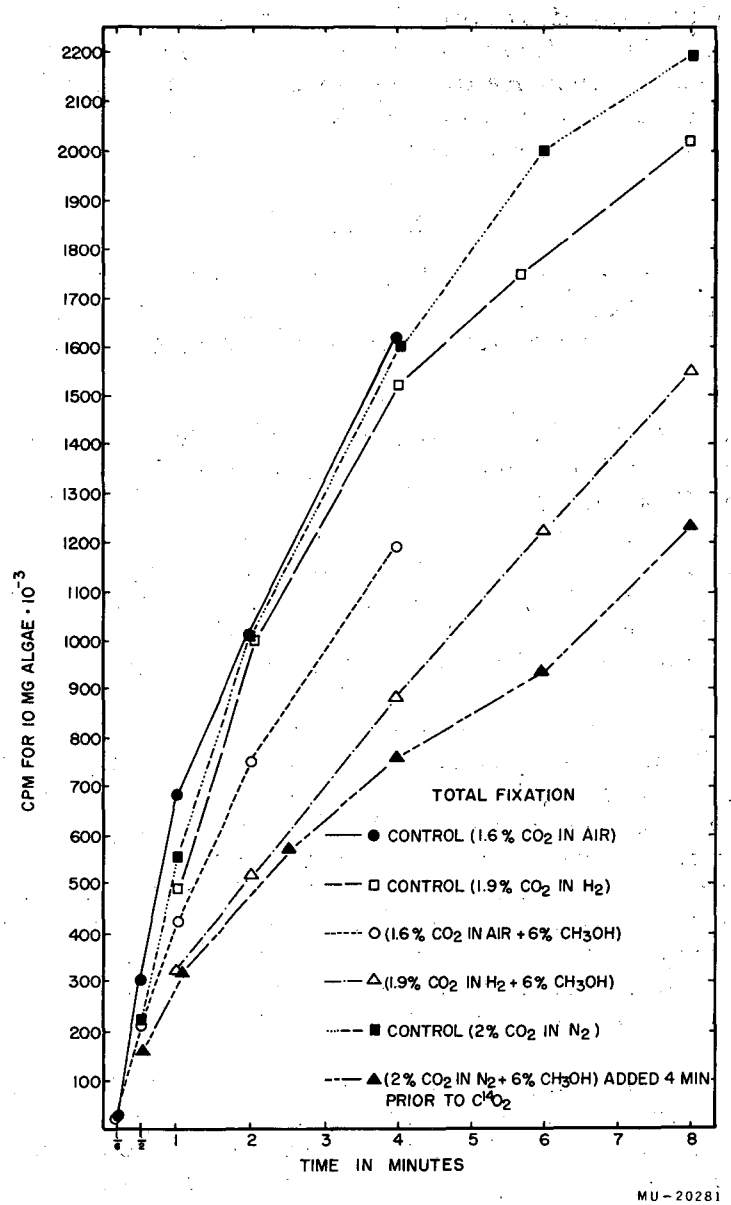

Fig. 39. Total fixation by Scenedesmus under a variety of conditions.

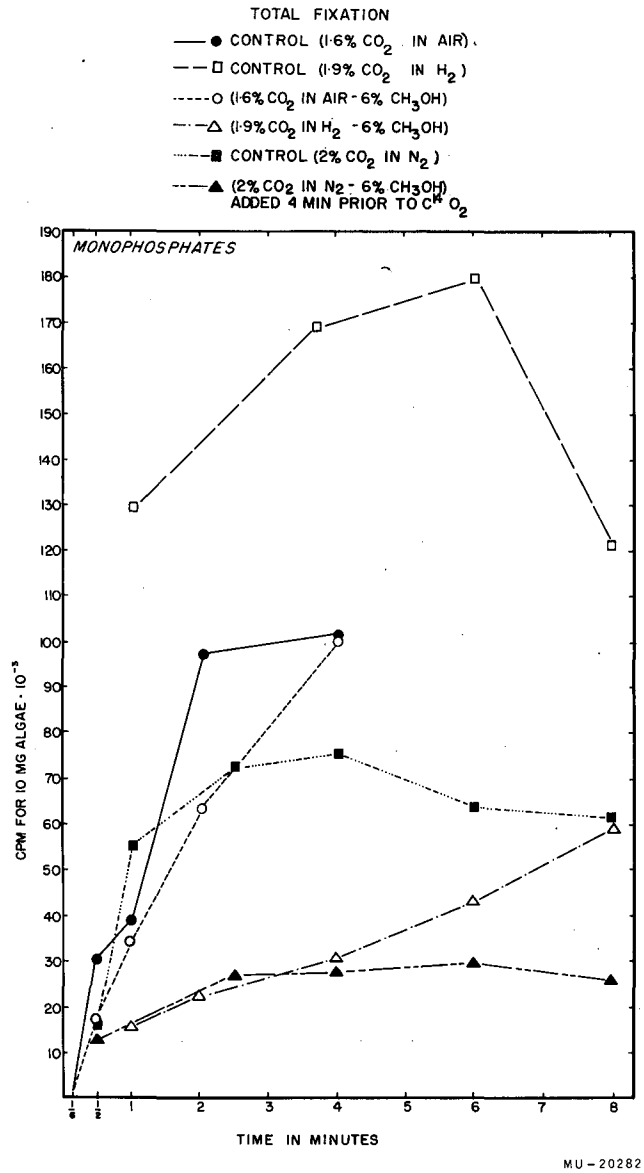

Fig. 40. Fixation in monophosphates by Scenedesmus under a variety of conditions.

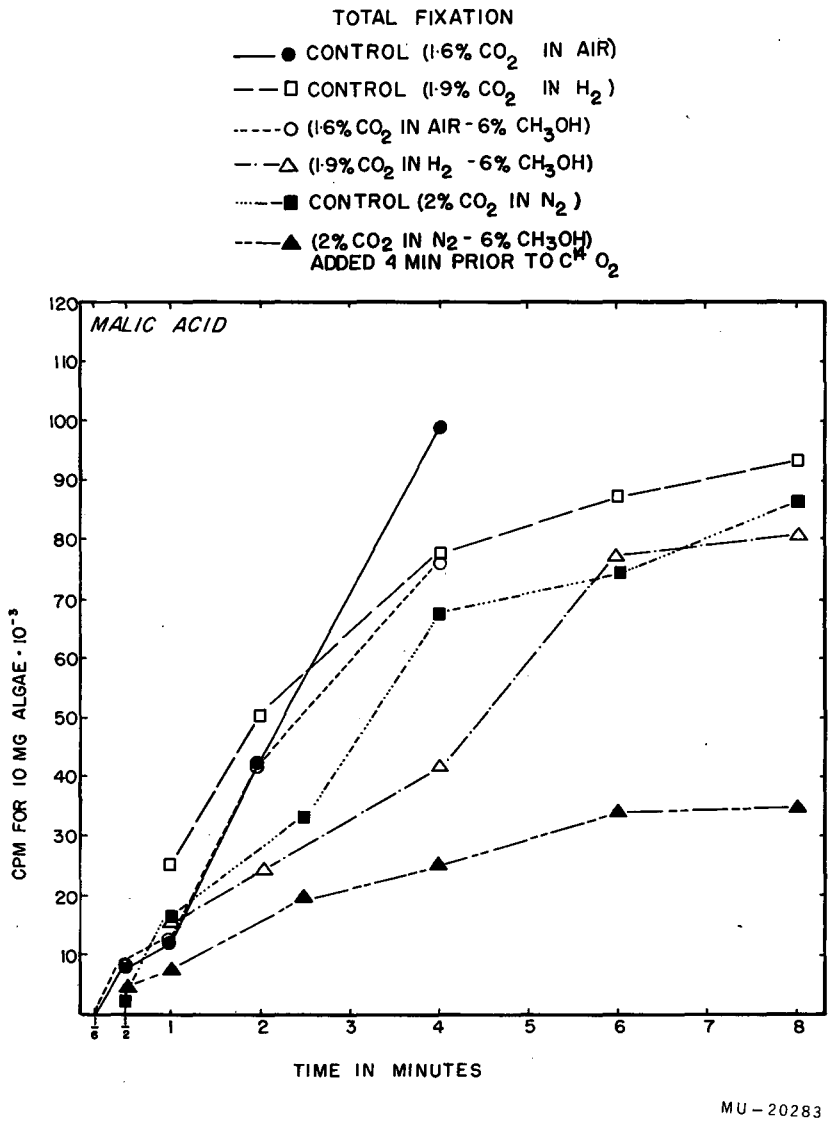

Fig. 41. Fixation in malic acid by Scenedesmus.

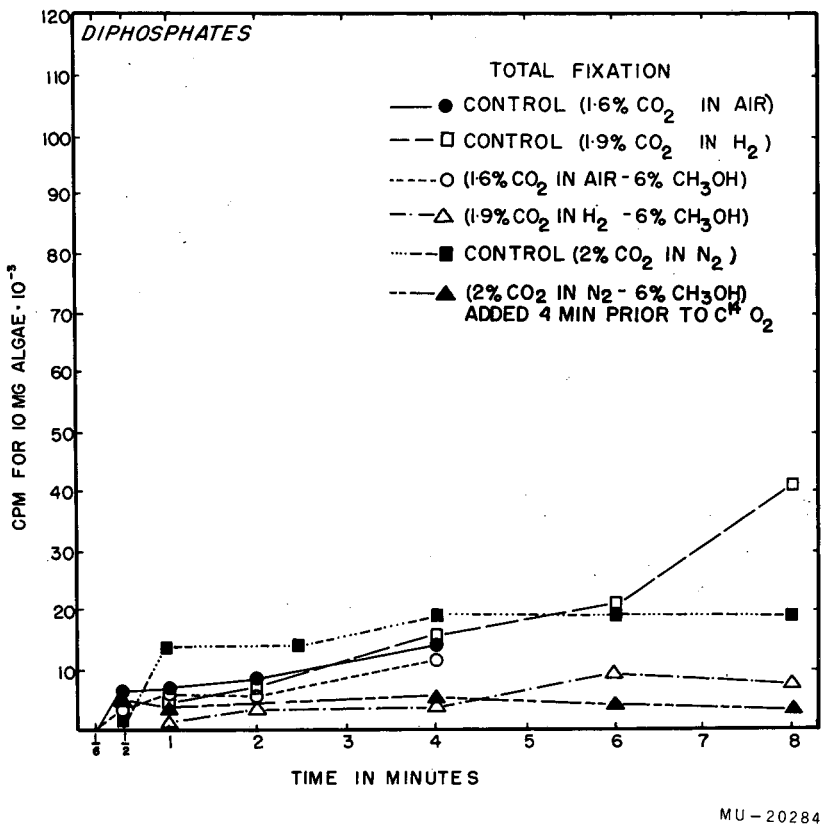

Fig. 42. Fixation in diphosphates by Scenedesmus.

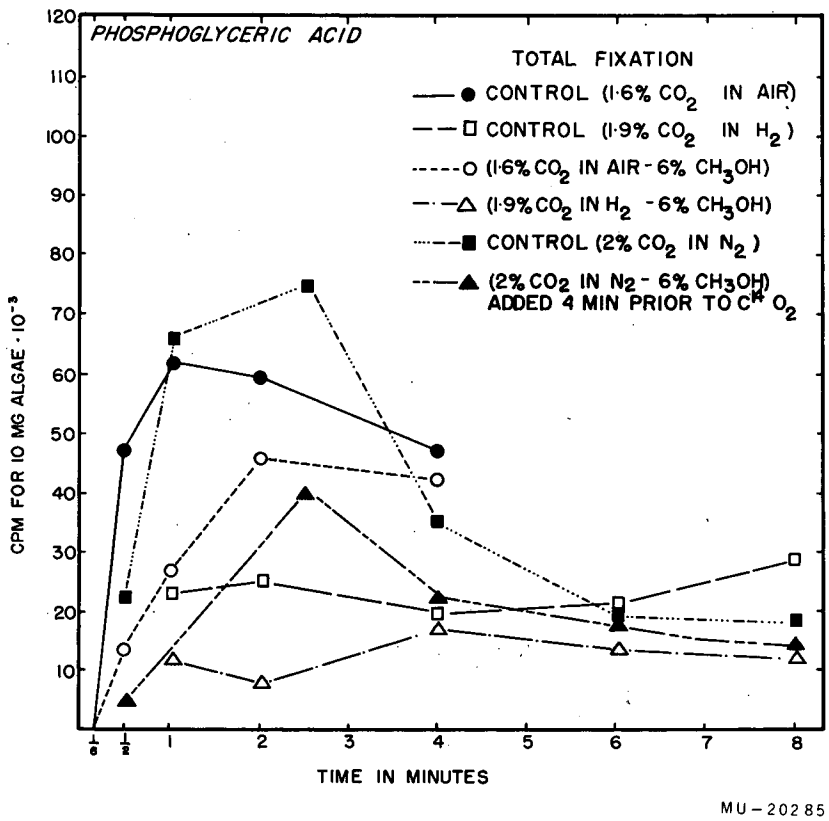

Fig. 43. Fixation in phosphoglyceric acid by Scenedesmus.

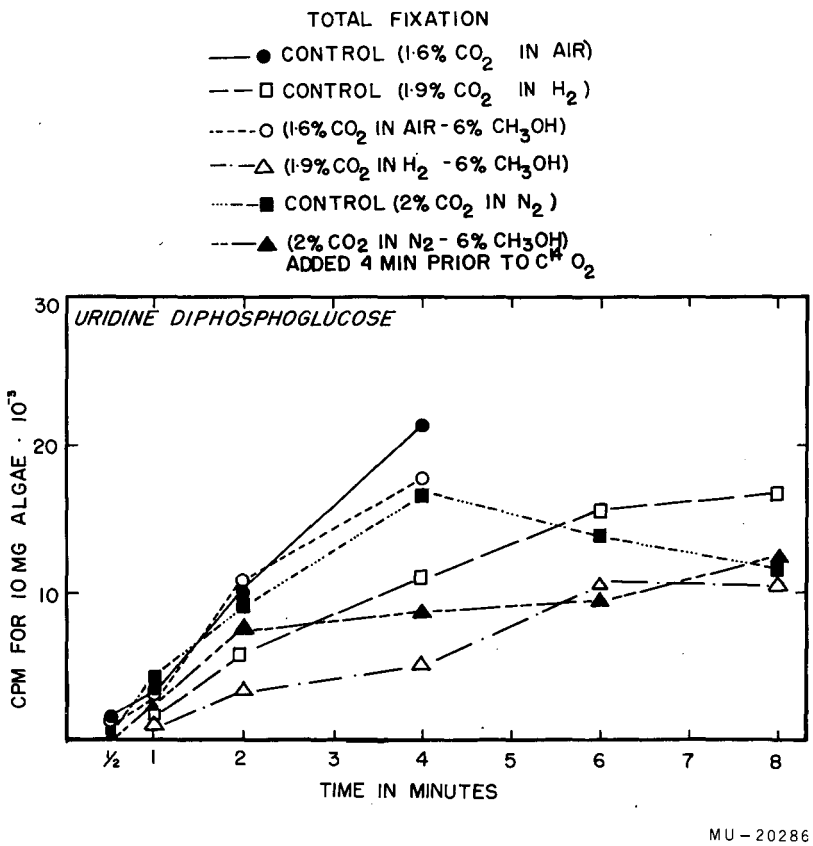

Fig. 44. Fixation in uridine diphosphoglucose by Scenedesmus.

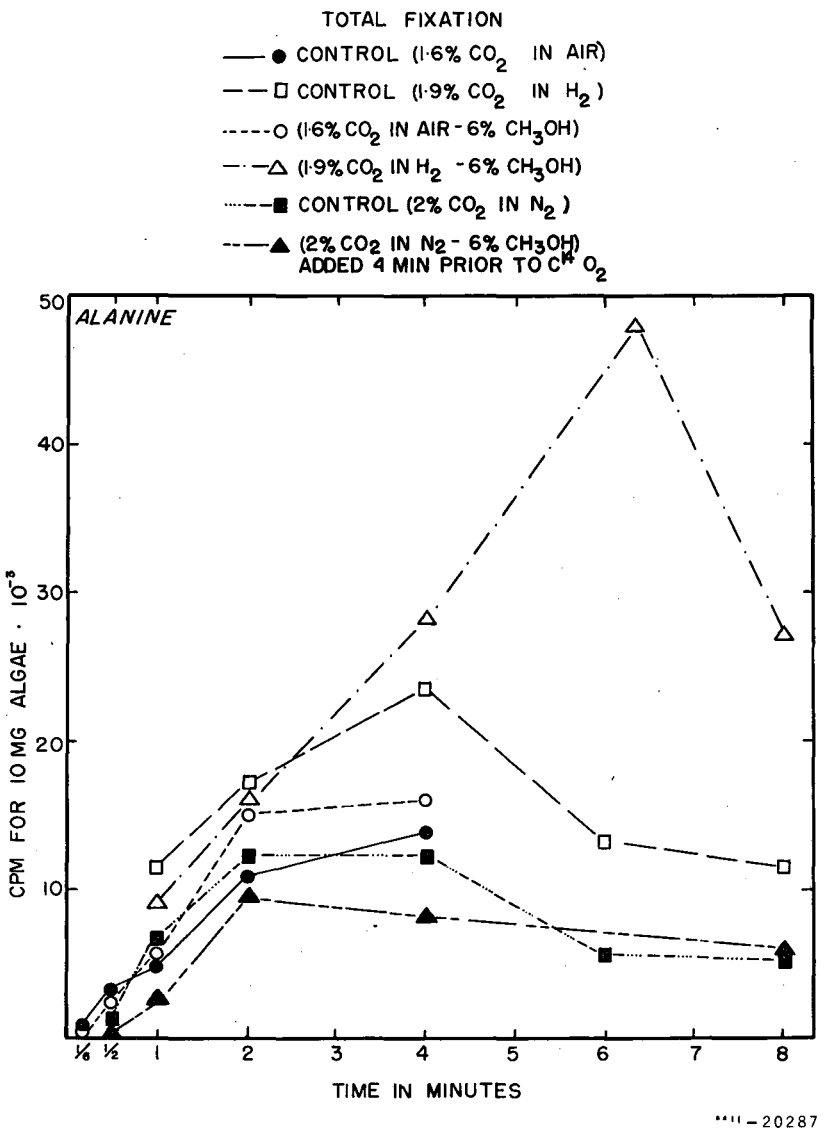

Fig. 45. Fixation in alanine by Scenedesmus.

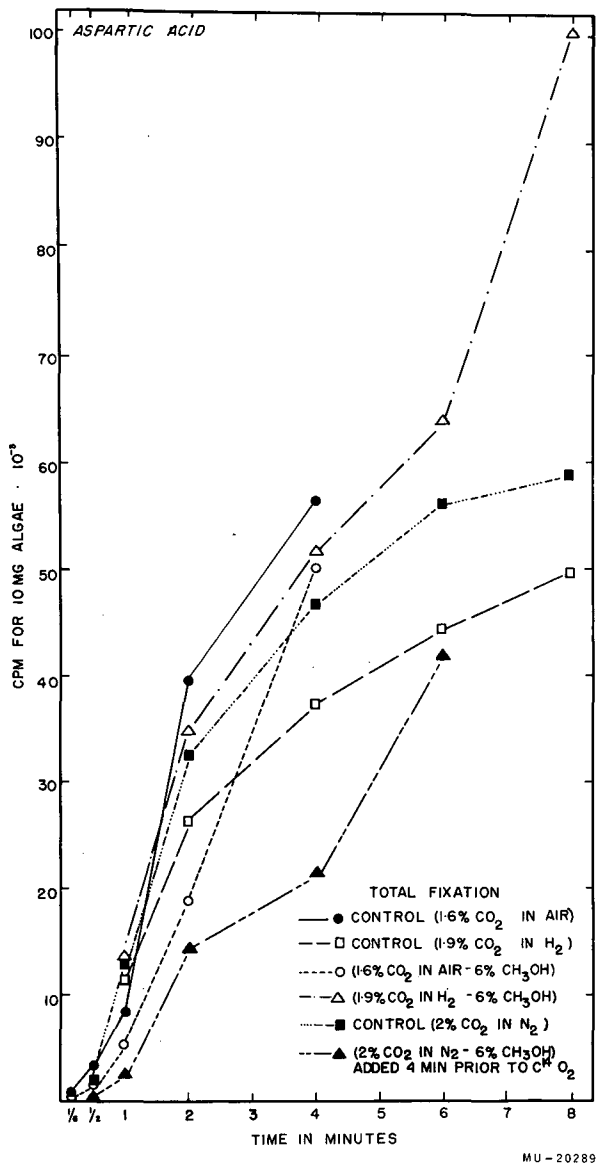

Fig. 46. Fixation in aspartic acid by Scenedesmus.

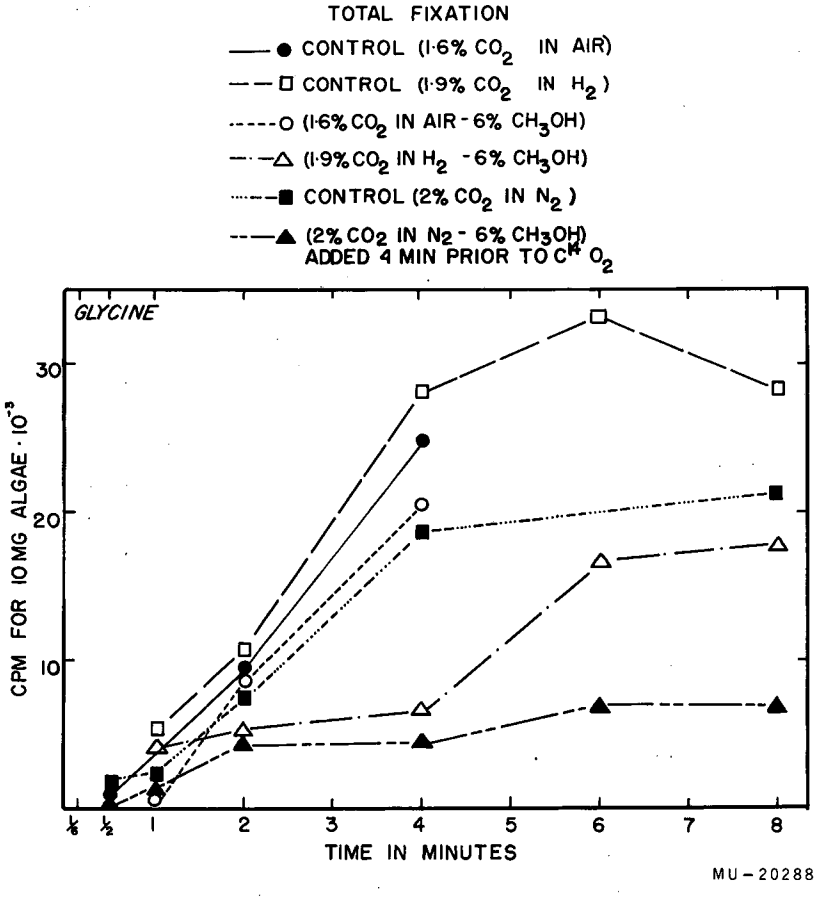

Fig. 47. Fixation in glycine by Scenedesmus.

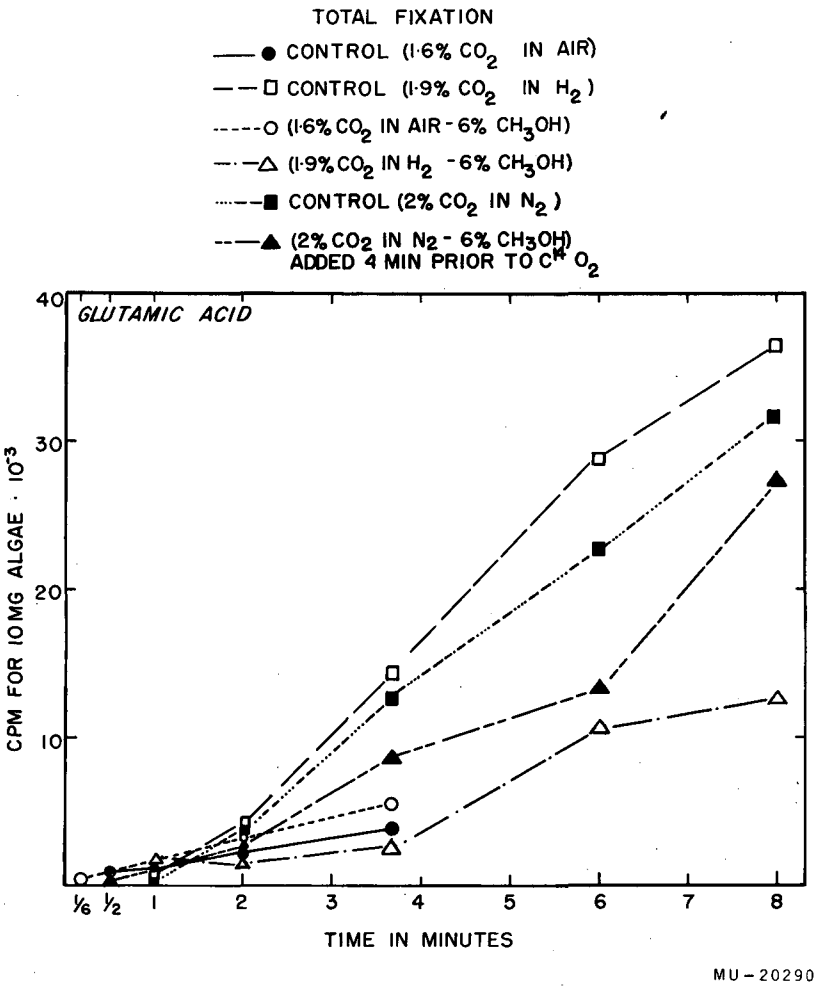

Fig. 48. Fixation in glutamic acid by Scenedesmus.

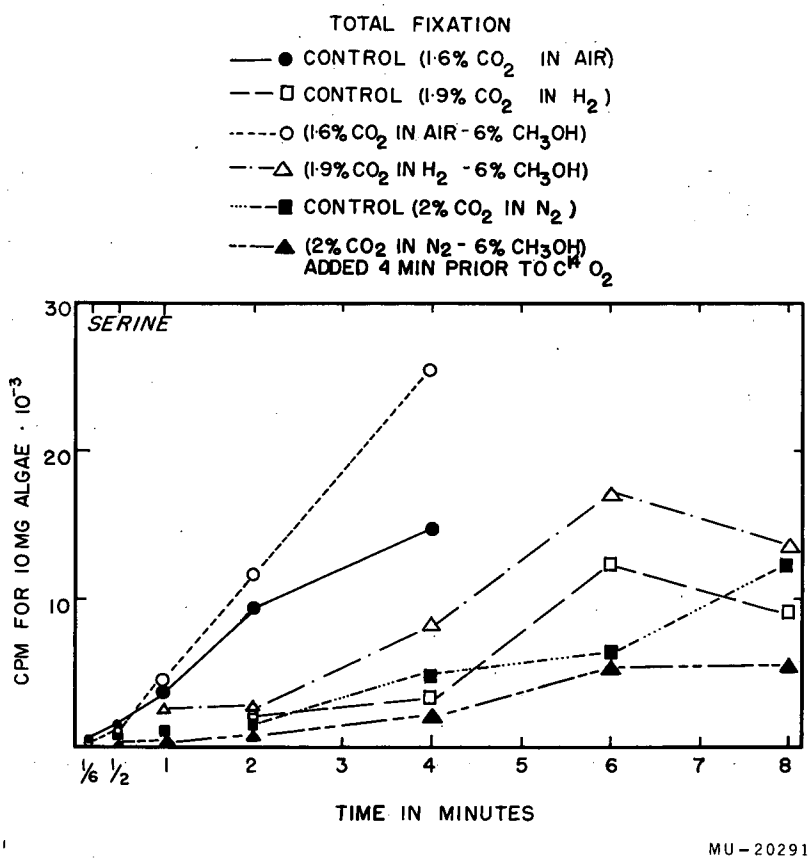

Fig. 49. Fixation in serine by Scenedesmus.

Table IX

Photosynthetic fixation in the absence of methanol: 1.6% CO_2 in air (activity in cpm for 10 mg of algae)

	Duration of illumination				
Compound	5 sec	0.5 min	1 min	2 min	4 min
DP	15	4,612	6,760	8,780	14,600
MP	105	34,226	38,695	97,670	111,602
PGA	952	46,952	61,460	59,510	97,391
PEP	32	3,488	4,085	2,980	3,036
UDPG	--	1,280	3,800	12,660	20,892
UDP	--	594	920	800	1,328
Carbamyl P	--	--	--	--	--
Malic Acid	56	6,888	10,075	42,680	99,177
Citric Acid	2	603	1,325	7,850	20,410
Fumaric Acid	--	--	330	1,240	2,949
Glycolic Acid	--	250	375	1,170	1,051
Succinic Acid	--	--	640	2,340	2,073
Aspartic Acid	14	3,352	8,430	39,490	56,385
Glutamic Acid	--	578	1,120	2,470	30,927
Glycine	--	149	1,085	9,730	24,630
Serine	4	586	3,840	9,430	25,790
Asparagine	--	--	--	3,800	13,578
Glutamine	8	277	520	4,790	22,440
Alanine	13	1,207	4,965	10,720	13,940
Tyrosine	--	--	--	--	--
Total	1,201	109,535	148,425	318,110	485,207

Table X

Photosynthetic fixation in the presence of methanol:
1.6% CO_2 in air with 6% methanol added 4 min prior to $C^{14}O_2$
(activity in cpm for 10 mg of algae)

	Duration of illumination				
Compound	5 sec	0.5 min	1 min	2 min	4 min
DP	14	3,815	6,128	5,893	11,090
MP	164	18,380	35,152	63,453	100,540
PGA	228	13,382	26,672	45,620	42,530
PEP	18	2,442	1,828	3,093	1,830
UDPG	--	960	2,876	8,980	15,120
UDP	--	371	224	753	1,810
Carbamyl P	--	--	--	--	--
Malic Acid	59	7,102	12,072	42,000	75,610
Citric Acid	6	120	2,588	4,283	11,080
Glycolic Acid	--	062	040	593	510
Fumaric Acid	--	--	228	1,806	1,830
Succinic Acid	--	--	412	1,226	1,270
Aspartic Acid	5	1,304	5,352	18,873	51,320
Glutamic Acid	13	369	596	3,026	5,600
Glycine	--	582	1,116	8,646	20,420
Serine	5	433	4,212	11,733	14,750
Asparagine	--	--	--	4,053	13,120
Glutamine	--	168	1,056	1,200	12,640
Alanine	33	2,413	5,620	18,413	12,660
Tyrosine	--	--	--	--	--
Total	548	51,903	106,172	246,647	393,730

Table XI

Photosynthetic fixation in the absence of methanol:
1.9% CO_2 in H_2 (activity in cpm for 10 mg of algae)

Compound	Duration of illumination (min)				
	1	2	4	6	8
DP	4,440	6,830	15,377	21,680	41,800
MP	129,635	70,130	172,683	181,960	133,250
PGA	22,905	25,020	18,942	21,180	29,250
PEP	525	800	932	1,540	1,950
UDPG	1,970	5,875	10,995	15,440	16,675
UDP	--	--	--	--	--
Carbamyl P	405	2,410	2,932	3,540	--
Malic Acid	25,420	50,520	76,919	87,180	92,950
Citric Acid	1,105	3,070	4,333	9,000	7,275
Fumaric Acid	--	920	1,683	1,300	1,475
Glycolic Acid	335	180	1,599	1,720	3,750
Succinic Acid	395	820	1,882	1,840	2,225
Aspartic Acid	11,470	26,180	37,151	44,200	49,525
Glutamic Acid	700	4,050	14,394	28,760	36,600
Glycine	4,110	10,880	28,255	33,120	28,125
Serine	510	1,710	3,665	12,380	8,950
Asparagine	820	3,120	7,530	16,720	16,725
Glutamine	1,820	4,100	8,863	17,940	21,475
Threonine	1,305	[illegible],840	2,698	4,240	4,450
Tyrosine	--	--	3,865	9,760	12,750
Valine	830	2,470	8,096	9,000	12,100
Leucine					21,375
Alanine	11,480	17,240	22,657	13,560	11,550
Total	219,235	237,160	441,619	534,220	556,850

Table XII

Photosynthetic fixation in the presence of methanol:
1.9% CO_2 in H_2 with 6% methanol for 4 min (activity in cpm for 10 mg of algae)

Compound	Duration of illumination (min)				
	1	2	4	6	8
DP	840	3,265	3,607	9,862	7,563
MP	15,324	23,475	31,505	51,947	64,190
PGA	11,800	8,375	7,555	13,362	11,495
PEP	1,168	280	166	3,250	385
UDPG	1,428	3,275	4,598	10,312	10,945
UDP	--	--	--	--	--
Carbamyl P	--	725	633	1,787	1,799
Malic Acid	15,976	24,870	42,033	77,837	80,867
Citric Acid	1,000	2,335	2,249	5,487	5,014
Glycolic Acid	256	125	83	525	499
Fumaric Acid	--	1,285	3,732	5,275	3,215
Succinic Acid	1,184	635	1,866	1,875	1,516
Aspartic Acid	13,600	34,935	51,621	63,662	100,176
Glutamic Acid	1,528	1,760	3,132	10,625	12,661
Glycine	5,548	5,210	6,713	16,700	17,776
Serine	4,416	2,605	8,913	16,912	13,194
Asparagine	--	930	5,980	10,500	3,681
Glutamine	1,300	6,550	14,702	24,725	22,407
Threonine	--	775	2,915	4,312	2,932
Tyrosine	856	1,025	3,706	8,962	14,434
Valine	2,328	860	2,948	7,125	6,064
Leucine	--	--	--	6,025	6,397
Alanine	9,072	16,260	28,158	47,800	27,072
Total	87,624	139,565	226,401	398,894	413,740

Table XIII

Photosynthetic fixation in the absence of methanol: 2% CO_2 in N_2 (activity in cpm per 10 mg of algae)

Compound	Duration of illumination (min)					
	1/2	1	2	4	6	8
DP	3,628	13,975	13,063	19,080	18,665	19,475
MP	15,084	55,567	75,341	76,000	63,816	62,550
PGA	21,150	60,685	27,593	34,800	23,220	19,925
PEP	1,703	6,024	5,598	3,760	2,466	2,250
UDPG	575	3,990	9,331	16,800	13,865	11,475
UDP	86	279	653	940	1,466	2,750
Carbamyl P	--	93	1,120	3,360	2,910	4,300
Malic Acid	2,291	16,178	32,525	67,080	74,215	86,400
Citric Acid	347	2,681	6,922	11,000	8,488	6,850
Glycolic Acid	--	85	226	1,920	2,888	11,200
Fumaric Acid	--	279	586	1,240	1,578	4,700
Succinic Acid	--	253	1,919	1,380	--	1,500
Aspartic Acid	1,476	12,874	31,120	46,480	42,040	58,475
Glutamic Acid	--	545	3,625	12,680	22,842	31,700
Glycine	289	2,247	7,771	19,280	11,110	21,100
Serine	55	479	1,386	4,840	6,044	12,175
Asparagine	--	--	--	--	--	1,350
Glutamine	281	1,881	4,079	10,140	16,265	28,200
Threonine	--	499	--	1,720	3,377	5,600
Tyrosine	--	253	1,120	2,160	1,844	1,450
Valine	--	372	2,053	1,880	7,332	7,500
Leucine	--	1,290	2,146	10,500	7,199	8,450
Alanine	1,005	6,650	12,434	12,040	5,533	5,750
Total	47,970	186,792	237,929	359,080	337,163	415,125

Table XIV

Photosynthetic fixation in the presence of methanol:
2% CO_2 in N_2 with 6% methanol for 4 min (activity in cpm for 10 mg of algae)

Compound	Duration of illumination (min)					
	0.5	1	2	4	6	8
DP	4,650	3,407	9,330	4,599	3,599	3,557
MP	12,270	41,281	27,255	27,597	30,072	25,987
PGA	22,920	17,596	39,901	20,220	18,155	14,856
PEP	1,300	1,771	2,832	1,777	1,720	1,514
UDPG	340	2,298	7,580	8,846	9,571	12,371
DUP	92	206	216		426	514
Carbamyl P	230	123	283		626	828
Malic Acid	2,785	6,853	19,825	24,086	33,885	34,541
Citric Acid	369	932	5,131	6,688	4,439	5,971
Glycolic Acid		86	208	422	693	600
Fumaric Acid	55	127	525	677	2,493	1,114
Succinic Acid		--	--	--	--	--
Aspartic Acid	795	2,790	14,161	21,209	55,693	20,427
Glutamic Acid	88	260	2,666	8,754	13,037	27,427
Glycine	453	746	4,773	4,377	6,958	5,628
Serine	103	140	833	2,088	5,519	5,343
Asparagine		--	--	783	746	1,757
Glutamine	202	689	3,907	5,733	17,062	15,242
Threonine		--	--	533	1,653	1,471
Tyrosine		--	--	1,111	2,359	3,914
Valine		220	1,024	1,577	2,799	5,257
Leucine		419	3,090	4,844	8,238	--
Alanine	1,045	2,844	9,271	8,377	16,209	5,342
Total	97,697	82,724	152,811	154,756	235,952	206,946

and the least in nitrogen. It is depressed by the methanol much more in nitrogen and in hydrogen than in air. For aspartic acid in air and nitrogen, the fixation is depressed by methanol, in hydrogen it is the opposite. In glutamic acid a similar inversion is observed in air, in which the fixation is greater in the presence of methanol. In serine the fixation is depressed by the methanol only in nitrogen, but not very much. In air and hydrogen, it is greater in the presence of methanol.

Discussion

The results described above, and obtained from the experiments performed under normal conditions of photosynthesis (the algae preilluminated in the presence of air and CO_2 and supplied with a great quantity of radioactive bicarbonate), do not always confirm the results of Ouellet and Lefrançois. As shown in the tables, the relative fixation in all compounds except glycolic acid is much greater in these experiments than in experiments by Lefrançois and Ouellet. The depression in such compounds as PGA, PEP, and aspartic acid is never so great as in the experiments of these authors. We confirm their results in which methanol increases the fixation in alanine, serine, and glutamic acid, but we have failed to confirm it in glycine. The fixation into glycolic acid, which--in the experiment by Ouellet and Lefrançois--is greater in the presence of methanol, and is reported by them as most sensitive to methanol, is small in the present experiments, and is depressed by methanol. In the N_2 experiments, the glycolic acid is more radioactive but is still depressed by methanol. To identify the unknown compound, we looked for a spot on the chromatograms in the area indicated by Ouellet and Lefrançois as "unknown compound." As shown in the pictures of their chromatograms (Fig. 38), the separation of the phosphates was not very good and the position of the unknown compound was that usually occupied by UDPG. The depression of fixation into this compound, which appeared very well separated on our chromatograms, was very small even in the presence of 10% methanol. The identification of this compound by the methods described in the experimental part show that it is UDPG.

To investigate the possibility that some of the differences between these experiments and those of Ouellet and Lefrançois might be caused by the absence of oxygen during the preillumination periods, an experiment was performed in which the algae were adapted in the atmosphere of CO_2 in nitrogen. Under such conditions, the total fixation of radioactive carbon as well as the fixation into malic acid, monophosphates, diphosphates, PGA, aspartic acid, glycine, and glutamic acid was depressed more in the presence of methanol than in air. The curves for alanine follow the same scheme in nitrogen as in air. UDPG in nitrogen shows, after 7.5 minutes, an even greater fraction of radioactivity in the presence of methanol than in air. In all these compounds, except in PGA and in serine, the fixation in both cases (in the absence and in the presence of methanol) is less in nitrogen than in air. Only PGA in the absence of methanol, and glutamic acid in both cases, show greater fixation in nitrogen than in air. These results in N_2 are more like those obtained by Ouellet and Lefrançois. They differ, however, in that methanol depresses fixation in N_2 while it increases fixation in air.

The effect of methanol was also investigated on the algae adapted to the mixture of CO_2 and hydrogen. It is known that Scenedesmus adapted in such an atmosphere utilizes the adsorbed hydrogen for the reduction of CO_2.[9] Benson found that unicellular algae produce a great variety of labeled compounds in 5-minute photoreduction in $H_2 + C^{14}O_2$.[10] In these experiments the influence of methanol on the algae preilluminated in the mixture of CO_2 and hydrogen was usually greater than in air. In MP, DP, glycine, and glutamic acid, the fixation in the absence of methanol is much greater in nitrogen than in air. In the presence of methanol it is much less. For PGA and serine, both fixation curves are lower in nitrogen than in air and for alanine, on the contrary, higher. For malic acid, the curve for the fixation in the presence of methanol in nitrogen lay below both fixation curves in air, but the control curve for malic acid (N_2 in the absence of methanol) lay between the two fixation curves in air. The aspartic acid shows, however, a much greater fixation in the presence of methanol in hydrogen than in air or nitrogen.

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[9] E. Rabinowitch, Photosynthesis and Related Processes, Vol. II, Part 2 (Interscience Publishers, New York, 1956), p. 1379.

[10] A. Benson and M. Calvin, Ann. Rev. Plant Physiol. 1, 25 (1950).

QUANTITATIVE ESTIMATION OF SMALL AMOUNTS OF AMINO ACIDS IN CHROMATOGRAPHIC ELUATES

David C. Smith

In the course of experiments on the formation of amino acids during steady-state photosynthesis in Chlorella, it was found necessary to develop a method of estimating the total amount of certain amino acids on chromatograms of extracts. The major difficulty of using a ninhydrin method for this purpose is that the paper may give a high and variable background color, which is presumably due mainly to traces of ammonia absorbed from the atmosphere or contained in the chromatographic solvents. It is particularly troublesome when the ninhydrin is in the reduced (and more sensitive) form necessary for the estimation of small amounts of amino acids.

It was found that this background color could be brought down to a low and relatively constant value if the eluates were made alkaline and then kept in vacuo for 15 minutes at room temperature with a gentle stream of ammonia-free air passed through them. Since accurate estimations with reduced ninhydrin must be made under buffered conditions, it is possible to use the alkaline component of the buffer system for the scrubbing of the eluate, and then the acid component can be added afterwards.

A method of utilizing this fact has been developed as follows. After amino acid spots on the chromatograms are located by autoradiography, the spots are cut out and each eluted separately into approximately 0.3 ml with water in graduated centrifuge tubes. One-tenth ml of 0.5 N NaOH is added and a stream of air bubbled gently through the tubes for 15 minutes in vacuo at room temperature (the air is first passed through a boric acid trap to remove ammonia). Then, 0.1 ml of 0.8 N citric acid is added to each tube to bring the pH to 5.0, followed by 0.2 ml of a KCN solution (8.33 ml in 0.01 M KCN in 250 ml methoxyethanol), and 0.2 ml of a 1.66% solution of ninhydrin in methoxyethanol. The total volume in each tube is made up to 0.9 ml with water, and after it is mixed well the solution is heated for 15 minutes in a boiling water bath (the mouths of the reaction tubes being stoppered with glass marbles to prevent losses by evaporation). The tubes and contents are cooled to 0°; then, 1.0 ml of 60% ethanol is added to each tube and the optical density at 570 mμ is determined in a Beckman spectrophotometer, using cells of 2.0 ml capacity. The color is light-stable for about 2 hours before addition of alcohol, and for about 1 hour afterwards. Under these conditions, for example, 1 μg glutamic acid is equivalent to an optical density (OD) change of 0.075. There is a linear relationship between OD and the amount of amino acid over the range 0.1 to 1.5 μg of amino nitrogen.

The background values for blank areas of a chromatogram should be determined (they are usually of the order of 0.070 OD/10 cm^2) and appropriate corrections applied to the amino acid values, depending on the area of the spots. It was found that the weights of the dried spots give a sufficiently accurate indication of their areas. The radioactivity of the spots is measured before and after elution, and it is generally found that 97% to 98.5% of the amino acid has been eluted; this has been confirmed by adding known amounts of glutamic acid to blank areas of chromatograms. Since fingerprints leave ninhydrin-positive marks on chromatograms, it is advisable to wear rubber gloves while handling papers at all stages of the chromatography.

The ninhydrin method described above has been modified from that described by Yemm and Cocking[1] by changing the volumes and concentrations of the reagents in order to increase sensitivity, and by including the method of scrubbing the eluate.

[1] E. W. Yemm and E. C. Cocking, Analyst 80, 209 (1955).

FURTHER STUDIES ON STERILITY PRODUCED IN MALE MICE BY DEUTERIUM OXIDE

Ann M. Hughes and Edward L. Bennett

We have previously reported that deuterium oxide in the drinking water of either male or female mice produces sterility.[1,2] An investigation of some of the conditions--with particular reference to time--of deuterium oxide treatment to produce sterile C_{57} male mice indicated that the sensitive phase of sperm production centered around the late prophase of meiosis.

The objective of the experiments reported here was to determine the phases of embryonic development of the mouse at which the lethal action of deuterium oxide on sperm is manifested. These investigations on embryonic growth initiated by sperm developed in D_2O have yielded additional evidence that D_2O severely damages the genetic material of developing sperm, with resulting sterility of the male mouse.

Methods and Results

"Dominant Lethal" Experiments

Male and female Swiss mice were used.[3] The fertility of the experimental and control male mice was determined by mating each with two or three female mice prior to the administration of D_2O to the experimental male mice. Only initially fertile mice were used in subsequent experiments. The fifteen experimental male mice were then supplied with drinking water containing 30% D_2O in lieu of tap water for the duration of the experiment. The males were 2-1/2 to 3 months old at this time. At weekly intervals, beginning 2 weeks after the first administration of D_2O, three (later four) normal virgin female mice (3 months old) were placed with each male. Ten control males supplied with tap water instead of deuterated water, but otherwise maintained under identical conditions, were mated at the same time. Each morning any female that had copulated (as indicated by the presence of a vaginal plug) was removed and housed in a separate cage. After one week, all females were removed and replaced with virgins. By this procedure the female mice received a minimal exposure to deuterated water, never longer than 1 week and, in many instances, only 1 or 2 days.

Sixteen or seventeen days after the vaginal plug was observed, or--if no vaginal plug was observed--16 days after removal from the male-mouse cage, the female mice were sacrificed. They were examined for viable embryos and for dead embryos or resorption sites (regressions).[4]

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[1] A. M. Hughes and M. Calvin, Science 127, 1445 (1958).

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

[3] These mice were obtained from Simonsen Laboratories, Gilroy, California.

[4] W. L. Russell, L. B. Russell, and A. W. Kimball, Amer. Naturalist 88, 269 (1954).

Results of "Dominant Lethal" Experiments

The results are summarized in Table XV. The data on the experimental and control mice in each group have been subdivided according to whether or not a vaginal plug was observed.

No increase in the number of regressions was observed. (The high percentage of regression obtained after six weeks of D_2O treatment is due to one female mouse that had seven regressions.) After 2 weeks of treatment with D_2O, no significant decrease in the percentage of pregnant females, or in the litter size, was noted. However, beginning 3 weeks after administration of D_2O a progressive decrease in both the number of embryos per pregnancy and the percentage of pregnancies was observed; after 6 weeks of treatment with D_2O, only 12% of the females became pregnant and the average number of embryos per pregnancy was 3.4. This compares with the over-all pregnancy ratio of 80% and 9.4 embryos per pregnancy observed in the control mice. Embryos per mating with the D_2O-treated mice had dropped to 5% of that of the controls after 6 weeks (0.4 embryo per mating, compared with 7.6 embryos per mating for the control).

Since each male mouse was mated with three (or four) female mice, the number of pregnant females obtained each week from each individual male can also be determined. This is shown in Table XVI. After 2 weeks of administration of D_2O, 13 of the 15 experimental mice had either 2 or 3 fertile matings (out of 3 possible), and only one male mouse had no fertile matings. (The following week he was fertile.) This may be compared with the observations made at 6 weeks, when only two of the 15 males had two fertile matings (out of four possible) and ten of the males did not have any fertile mating.

The results of this experiment strongly suggest that sperm produced in the presence of D_2O are either so defective that they are unable to penetrate the egg[5] or that when they enter the egg fertilization occurs, but damaged genetic material leads to early death of the fertilized mouse egg.

Observations of Development of Mouse Eggs Fertilized by D_2O-Treated Males

Eight male Swiss mice 3 months old (from the Lawrence Radiation Laboratory colony) were provided with drinking water containing 30% D_2O in lieu of tap water for 5-1/2 to 6 weeks. The male mice were placed at midnight in cages with virgin female mice in estrus. At 10:00 a.m. the following day, the females were removed and the presence of vaginal plugs determined. Thus, the time of fertilization was known within 10 hours. Normal control mice were mated in the same fashion. At the desired time after copulation, the female mice were sacrificed, and the eggs flushed from each oviduct with 0.9% NaCl and observed under magnification up to 250× after staining with acridine orange (1 to 40,000 in 0.9% NaCl and 0.01 M phosphate buffer, pH 7.4). The results are summarized in Table XVII.

[5] The presence of vaginal plugs indicates that copulation occurred by D_2O-treated animals. Sperm motility is presumably not an important factor in the transport of sperm through the uterus and ovarian tubes. (G. D. Snell, in Biology of the Laboratory Mouse (Dover Publications Inc., N. Y., 1956), p. 78).

Table XV (continued)

Effect of 30% D_2O in drinking water on fertility of male mice

	Time of treatment prior to mating (weeks)								
	D_2O-treated males (15)				Control males (10)				
Combined	2	3	4	6[a]	2	3	4	6[a]	Totals 2 to 6
No. of females	45	45	44	60	30	30	30	38	128
Total no. pregnant	34	28	17	7	25	24	23	31	103
Percent pregnant	76	62	39	12	83	80	77	82	80
Total no. embryos	330	183	108	24	225	213	251	284	973
Total no. regressions	26	24	13	8	27	31	15	29	102
Percent regressions	7.3	11.6	10.7	25	10.7	12.7	5.6	9.3	9.5
Embryos/pregnancy	9.7	6.5	6.4	3.4	9.0	8.9	10.9	9.2	9.4
Embryos/mating	7.3	4.1	2.5	0.4	7.5	7.1	8.4	7.5	7.6

[a] Four females/male

Table XV

Effect of 30% D_2O in drinking water on fertility of male mice

	Time of treatment prior to mating (weeks)								
	D_2O-treated males (15)				Control males (10)				Totals 2 to 6
	2	3	4	6[a]	2	3	4	6[a]	
Mice with vaginal plugs									
No. of females	24	23	23	17	16	11	11	8	46
No. pregnant	23	16	11	4	15	9	11	8	43
No. embryos	246	107	66	19	148	75	132	87	442
No. regressions	15	12	6	0	15	16	9	10	50
Percent regressions	5.7	10.1	8.3	0	9.2	17.6	6.4	10.3	10.2
Embryos/pregnancy	10.7	6.7	6.0	4.8	9.9	8.3	12.0	10.9	10.3
Mice without vaginal plugs									
No. of females	21	22	21	43	14	19	19	30	82
No. pregnant	11	12	6	3	10	15	12	23	60
No. embryos	84	76	42	5	77	138	119	197	531
No. regressions	11	12	7	8	12	15	6	19	52
Percent regressions	11.6	13.6	14.3	75	13.5	9.8	4.8	8.8	8.9
Embryos/pregnancy	7.6	6.3	7.0	1.7	7.7	9.2	9.9	8.6	8.9

Table XVI

Frequency of fertile matings of D_2O-treated male and normal female mice: number of males associated with N pregnant females[a]

Duration of treatment (weeks)	Control male mice (10) Number (N) of pregnant females per male					D_2O-treated male mice (15) Number (N) of pregnant females per male				
	4	3	2	1	0	4	3	2	1	0
2	-	5	5	0	0	-	7	6	1	1
3	-	4	6	0	0	-	4	6	3	2
4	-	4	5	1	0	-	2	5	2	6
6	4	4	0	1	0	0	0	2	3	10

[a]Three female mice were mated with each male except at 6 weeks, when four female mice were mated with each male.

Table XVII

Stage and condition of eggs from mice fertilized by normal or D_2O-treated males

	No. females	Time after copulation (days)	Females without eggs	Condition of eggs	Stage of eggs (No. of cells) 1	2	3	4	8 or more	Total eggs
Control males	5	1-1/2 to 2	1	N[a]	3	4	3	2	25	37
				D[a]	0	0	0	0	0	0
D_2O-treated male	3	1-1/2 to 2	0	N	23	4	1	0	0	28
				D	1	1	0	0	0	2
Control males	5	2-1/2 to 3	1	N	0	0	0	0	28	28
				D	1	1	0	0	0	2
D_2O-treated males	9	2-1/2 to 3	2	N	9	4	0	0	4	17
				D	1	6	2	1	33	43

[a]N = normal, D = degenerating

In general, 55 to 65 hours after fertilization, most control mice had numerous eggs in the multicell stage (8 to 16 cells). Relatively few two- or four-cell eggs, or disintegrating eggs, were observed. However, a high frequency of abnormal eggs was observed in females mated with D_2O-treated mice; many were one, two, or four cells. The cells were often not of uniform size, and in many instances they were not congruent. A few of the aberrant cell types are shown in Fig. 50. These aberrant cells can be compared with examples of degenerating cells found in a study by Lewis and Wright.[6]

After the experimental males had been provided with 30% D_2O for 7 weeks, they were returned to ordinary water. Two weeks later these mice were again mated with normal females, and the eggs were examined approximately 60 hours after copulation. Again, a high frequency of degenerating eggs was observed. However, the earlier work[2] showed that after the D_2O-treated male mice are returned to tap water for 3 weeks or longer, they are again normally fertile mice.

Discussion

A reduced number of litters and fewer mice per litter are obtained when male Swiss mice that have been maintained on 30% D_2O in lieu of tap water are mated with normal female Swiss mice. Nearly complete sterility is obtained when Swiss male mice are maintained on 30% D_2O for 6 weeks. At this time, although many broken and nonmotile sperm are present in the vas deferens, 20,000 to 30,000 motile sperm were observed in each vas deferens of two sterile male mice which were examined, confirming a similar observation on C_{57} mice.[2] It is extremely unlikely that the degeneration of the eggs could have resulted from the mere presence of 20% to 30% D_2O in the sperm, since a sperm constitutes an extremely small fraction (much less than 0.1%) of the total mass of a fertilized egg. In addition, the gross sterility observations have shown that sterile matings result from male mice that have been returned to normal water for at least 2 weeks after a 2-week treatment with 30% D_2O.[2] In our study we found degenerating eggs when male mice that had been on D_2O for 7 weeks and then returned to normal water for 2 weeks were mated with normal females. The sperm of mice returned to normal water for 2 weeks certainly have very little deuterium in them.

Our experiments have not shown that the sperm entered the eggs. The observation of motile sperm would suggest that the sperm would be capable of entering the eggs. Such sperm, if abnormal in genetic complement, could lead to subsequent divisions that would be abnormal and that, in many instances, would not proceed past the one- to four-cell stage. Experiments to determine whether the sperm do enter the egg, and whether fusion of the pronuclei does occur, are in progress.

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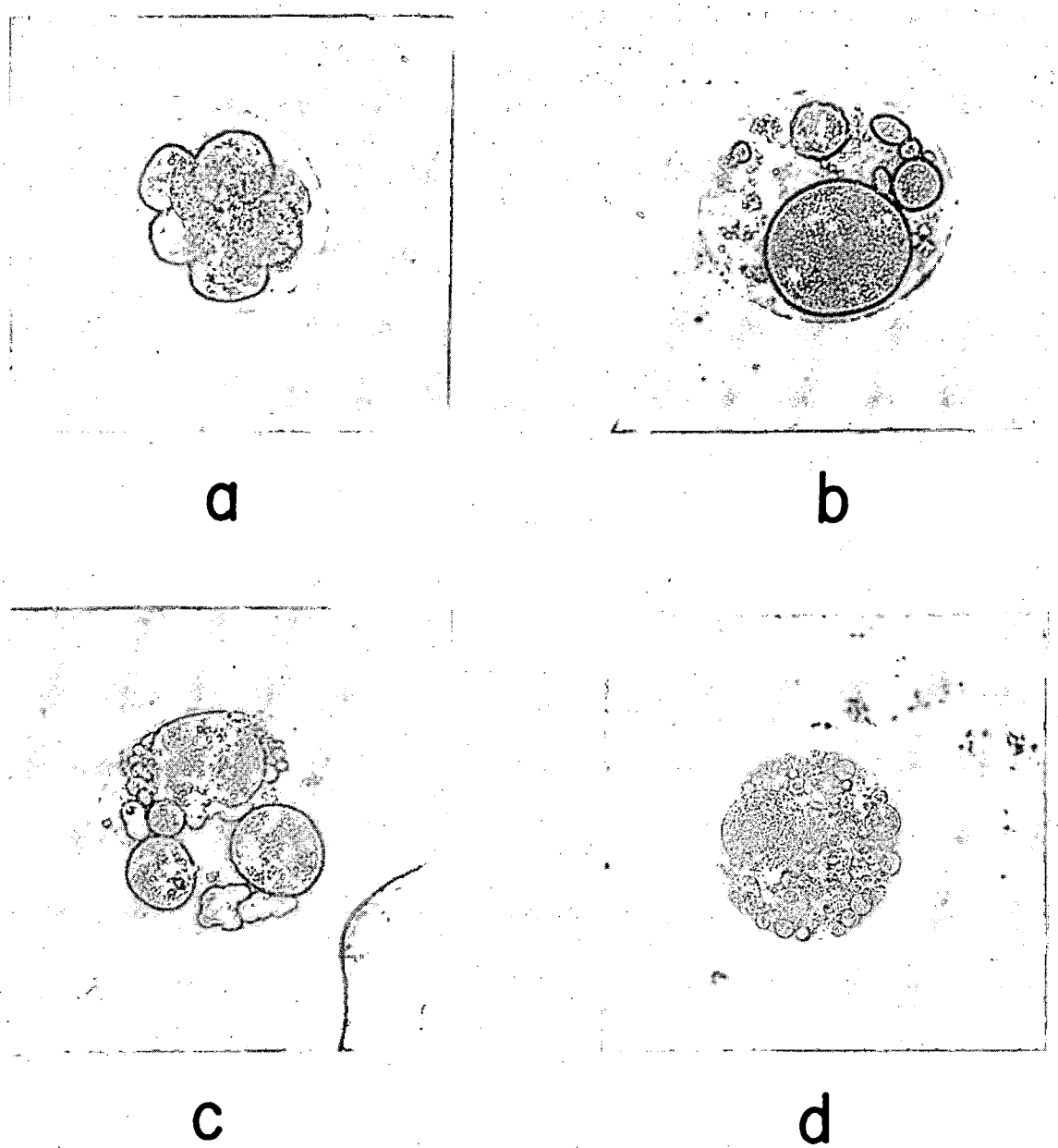
[6] W. H. Lewis, and E. S. Wright, Contributions to Embryology, Carnegie Institution of Washington, 25, 115 (1935).



ZN-2570

Fig. 50. Photomicrographs (× 250) of eggs obtained 2 to 2-1/2 days after copulation. Upper left is an example of a normal 8-cell egg. Upper right and both lower pictures are examples of abnormal cell types resulting from mating of D_2O-treated males with normal female mice.

Our experiments indicate no increase in embryonic deaths after implantation. This is in marked contrast to the effects of radiation in which a large increase in such deaths, termed "dominant lethal mutation," occurs.[3] This suggests that the damage to the genetic material caused by D_2O differs from damage caused by radiation. Previous experiments indicated that the phase of sperm production most sensitive to D_2O is the meiotic phase. Sperm that have undergone meiotic division in the presence of D_2O during their genesis are not capable of producing offspring even though the animal is not subsequently supplied with D_2O.

At this stage of sperm production, chromosomal reorganization occurs. It is suggested that the presence of deuterium markedly affects this process, principally by changes in the forces of association of macromolecules. Motile sperm are obtained, but the genetic material is so abnormal that although the egg may be fertilized, its death soon follows. Experiments are in progress to further characterize changes in the genetic material of sperm resulting from the presence of D_2O.

Acknowledgment

We are grateful to Dr. T. P. Lin, Department of Anatomy, University of California Medical School, San Francisco, California, for demonstrating the technique for flushing the mouse eggs from the oviducts. Dr. Lin and Dr. Laurel Glass (of the same department) also assisted us in interpreting the photomicrographs of the eggs.

FURTHER STUDIES ON THE EFFECTS OF D_2O ON DROSOPHILA MELANOGASTER

Ann M. Hughes

In a previous report we presented data indicating that 20% D_2O in the media on which male or female drosophila were grown increased the occurrence of offspring resulting from nondisjunction in the treated parent.[1] Subsequent experiments designed to duplicate these results have failed to do so. In an examination of factors that might cause this discrepancy, it was found that the stock D_2O used in the first series of experiments contained a relatively high concentration of tritium (32.6 ± 1% μC/liter), while that used in the second series contained only 3.75 ± 4% μC/liter.

In order to determine whether the tritium content of the D_2O was the critical factor in the increased occurrence of nondisjunction reported in the first series of experiments, the experiment involving nondisjunction in the male has been repeated. The control flies were raised on media made with D_2O containing 4.35 μC H^3 per liter. The experimental flies were raised on media made with D_2O containing 50.5 ± 5% μC H^3 per liter. The same stock of drosophila was used as had been used in the previous male-nondisjunction experiments. Results are shown in Table XVIII.

Table XVIII

Study of effects of tritium in nondisjunction experiment

	Total offspring	Unusual[a] females	Unusual males	Unusual females plus males	% Unusual flies
Control ("cold" D_2O)	15476	8	9	17	0.11
Experimental ("hot" D_2O)	16304	6	22	28	0.17

[a] "Unusual" refers to flies resulting from nondisjunction.

The differences in total numbers of unusual offspring is not quite significant, but suggests a trend. However, the difference in occurrence of unusual male offspring is significant at about the 2% level and seems to substantiate our earlier findings.[1] We suggested then that the increase in unusual males without a concomitant increase in unusual females might indicate a loss of part or all of the Y chromosome, rather than nondisjunction. This concept of breakage and loss of parts of the Y chromosome was further borne out by our experiments in which both arms of the Y chromosome carried markers.[2]

This most recent experiment appears to indicate that the amount of tritium in the "hot" D_2O is enough to cause genetic damage, even though such a quantity of tritium would expose each fly to less than 0.05 r irradiation. We are repeating the male-nondisjunction experiment in an attempt to improve the statistical significance of our results.

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

[2] Ann M. Hughes, in Bio-Organic Chemistry Quarterly Report, UCRL-9135, March 1960, p. 58.

INVESTIGATION OF POSSIBLE BIOCHEMICAL DIFFERENCES CORRELATED WITH ETHANOL PREFERENCE IN MICE

Edward L. Bennett and Marie Hebert

(in collaboration with David A. Rodgers and Gerald E. McClearn, Department of Psychology, University of California)

In an investigation of the genetic, physiological, and environmental aspects of voluntary ethanol consumption of mice by McClearn and Rodgers,[1] marked individual and genetic differences have been observed. The preference for ethanol can be systematically altered by manipulation of genotype. C_{57} mice showed higher mean preference for 10% ethanol than for water in a free-choice situation than five other strains of mice that were tested. The preference for 10% ethanol over water has been shown to be genetically transmissible. First-generation animals derived from crosses of the high-ethanol-preference C_{57} strain and the low-preference strains showed a mean preference intermediate between that of the parent strains and somewhat below the midparent values. When offered a choice of water or ethanol ranging in six concentrations from 2.5% to 15% in increments of 2.5%, the BALB/c and A/3 strains showed almost total avoidance of all ethanol concentrations, and the A/2 mice showed a slight acceptance of very low ethanol concentrations. The C3H/2 showed relatively uniform preference for all solutions containing 10% or less ethanol, and with time showed a progressive shift towards the higher concentrations of ethanol.

These and other experiments suggest that a genetic and therefore a biochemical difference in the rate or route of utilization of ethanol exists in mice. Ethanol is metabolized primarily in the liver. The first step in the metabolism of ethanol is oxidation to acetaldehyde by the diphosphopyridine nucleotide (DPN) linked enzyme alcohol dehydrogenase (ADH). Subsequently, acetaldehyde may be converted to pyruvate or acetate and eventually enter the Krebs cycle and be metabolized to carbon dioxide.

We therefore have investigated two aspects of the metabolism of ethanol in these strains of mice: (a) the activity of ADH in the liver *in vitro*, and (b) *in vivo* experiments on the over-all rate of ethanol-1-C^{14} oxidation. In the first type of experiment (described in more detail below) it was found that the activity of ADH per g of liver was similar for five of the six strains tested. However, the ADH activity of the C_{57} strain was about 50% higher. A second method of expressing the data is to consider the total activity of ADH (by multiplying the ADH activity/g liver by the liver weight), and a third measure can be obtained by dividing this latter value by the mouse weight to obtain an indication of the ability of the mouse to oxidize ethanol based upon total body weight. By all these measurements, the C_{57} mice are high relative to the other strains tested (Table XIX). In addition, when the ADH-activity per unit of body weight is compared with the proportion of 10% ethanol solution to total liquid intake when the mice are given a choice between 10% ethanol and water, a positive correlation is obtained (Fig. 51).

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[1] G. E. McClearn and D. A. Rodgers, Quart. J. Studies Alc. 20, 691-695 (1959).

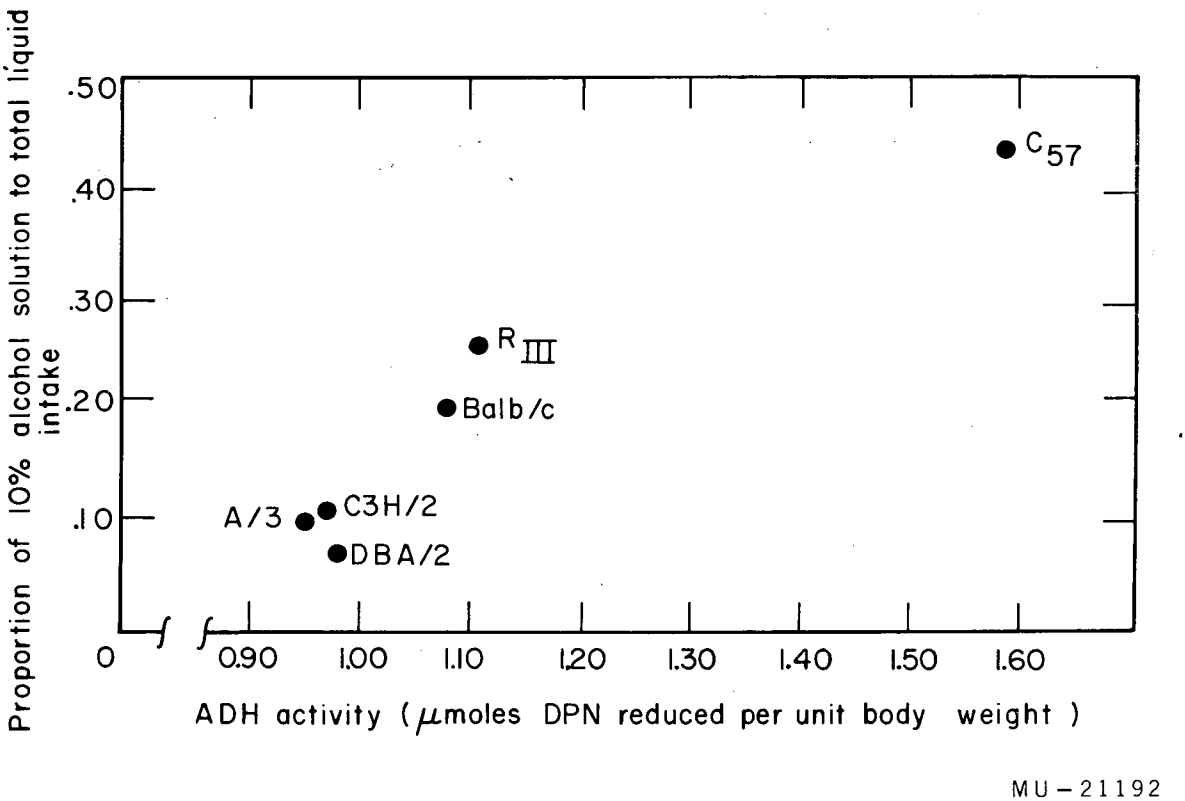

Fig. 51. A comparison of liver ADH to ethanol preference of six strains of mice.

Table XIX

Rate of oxidation of EtOH by mouse liver homogenates

Strain	Number of mice	EtOH oxidized (μM/min/g liver)	EtOH oxidized (μM/min/ liver)	EtOH oxidized (μM/min/g mouse)
DBA/2	5	1.99±0.20[a]	3.14±0.36	0.098±0.009
A_3	6	2.02±0.25	2.98±0.30	0.095±0.009
$C_3H/2$	6	1.91±0.16	3.54±0.36	0.097±0.008
Balb/c	6	2.08±0.17	3.72±0.40	0.108±0.006
R_{III}	6	1.85±0.13	4.11±0.37	0.111±0.000
C_{57}	6	3.00±0.08	4.61±0.83	0.159±0.013

[a] Average ± $\bar{\sigma}$; $\bar{\sigma} = \sqrt{\frac{\Sigma X^2}{N-1} - \frac{(\Sigma X)^2}{N(N-1)}}$

However, when the abilities of the C_{57} and DBA mice (strains which differ greatly in ADH activity) to metabolize C_2H_5OH-1-C^{14} were determined, the maximum rate of metabolism and the percentage of the administered dose metabolized by these strains were similar under a variety of test conditions (Tables XX and XXI).

These test conditions included dosages ranging from 0.1 μl of ethanol per g body weight to 4 μl of ethanol per g body weight. At the highest dosage employed, the animals were comatose. As expected, the smaller the amount of ethanol, the sooner the maximum rate of oxidation is obtained. With increasing amounts of ethanol, the maximum absolute rate of oxidation (averaged over a 5-minute interval) increases (Fig. 52). The increase is small above a dose rate of 1 μl of ethanol per g weight, indicating that ethanol is not rate-limiting under these conditions. No significant strain differences were found. Indeed, if any difference does exist, it is in the direction of a higher maximum rate of excretion of ethanol-1-C^{14} as $C^{14}O_2$ by the DBA mice.

Thus, the in vitro test and the in vivo test give contradictory results, since by the in vitro determination of ADH activity, the C_{57} mice have a higher ability to oxidize ethanol than do the DBA mice, whereas by the in vivo test both strains actually oxidized ethanol to carbon dioxide at equal rates. Further genetic experiments are in progress with DBA and C_{57} mice to determine if ethanol preference and ADH activity are correlated. If positive results are obtained, other enzyme systems concerned with ethanol metabolism will be studied.

Methods

Alcohol dehydrogenase activity can be ascertained by the rate of reduction of diphosphopyridine nucleotide (DPN) and the consequent increase in absorption at 340 mμ under standard conditions. Several methods have been described.[2-4] These methods differ primarily in the type and pH of the buffer used. One method utilizes a trapping agent, semicarbazide, to react with the acetaldehyde formed.[5,6] We compared the rate of oxidation of ethanol by a mouse liver homogenate by these several methods, and found the latter method with the trapping agent gave appreciably higher rates of DPN reduction (Table XXII).

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[2] E. Racker, J. Biol. Chem. 184, 313 (1950).

[3] H. Theorell and B. Chance, Acta Chem. Scand. 5, 1127 (1951).

[4] A. Nason, N. Kaplan, and S. Colowick, J. Biol. Chem. 188, 397 (1951).

[5] H. Theorell and R. Bonnichsen, Scand. J. Clin. Lab. Invest. 3, 58 (1951).

[6] The buffer used by Theorell and Bonnichsen is described incorrectly by E. Marshall, Jr., and W. Fritz, J. Pharmacol. Exptl. Therap. 109, 431 (1953), and results in a final pH of 12.

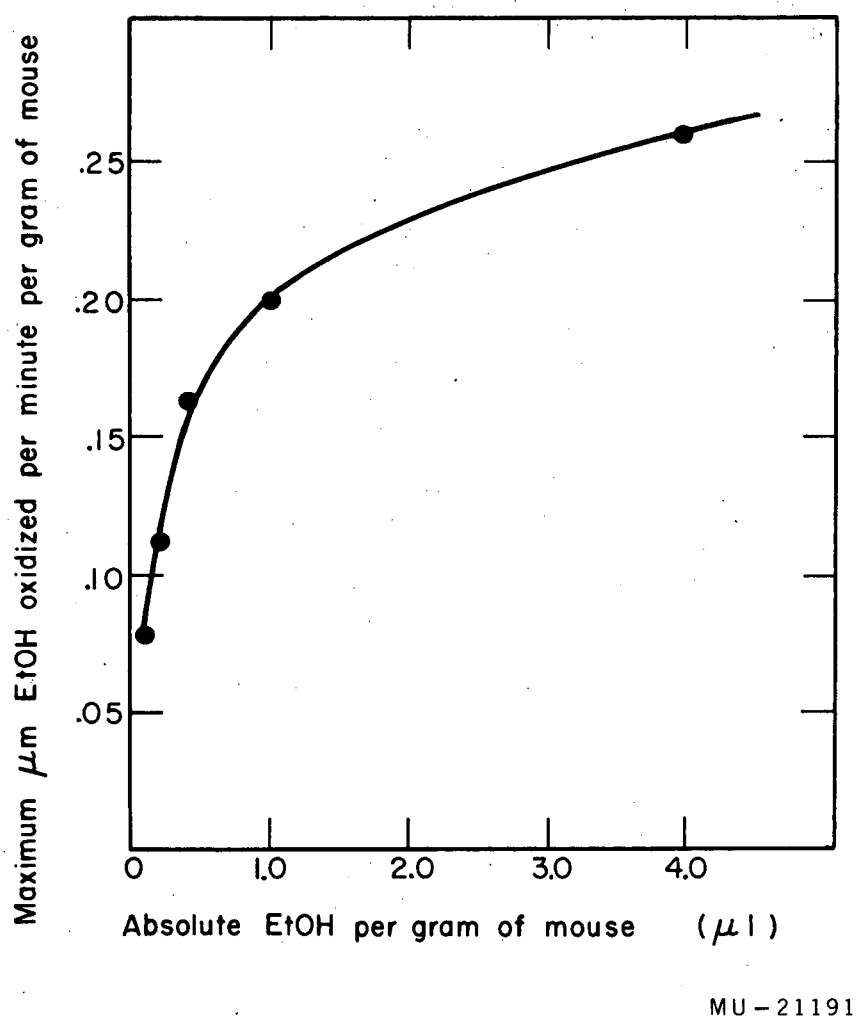

Fig. 52. Maximum rate of ethanol oxidation as a function of dosage in mice.

Table XX

Cumulative excretion of $C^{14}O_2$ from injected EtOH-1-C^{14} at various times (%)

Strain	No.	Time interval (min)					
		0 to 10	0 to 20	0 to 40	0 to 60	0 to 120	0 to 180
DBA	11	2.36 ±0.49	9.50 ±2.07	28.35 ±5.41	49.14 ±7.94	73.45 ±6.88	86.43 ±4.15
C_{57}	13	2.00 ±0.63	8.50 ±2.27	26.12 ±4.98	45.24 ±7.07	70.93 ±7.33	85.80 ±5.06

Ethanol-1-C^{14} administered intraperitoneally as 10% ethanol. One μl absolute ethanol used per gram body weight of mouse.

Table XXI

Effect of dosage on maximum rate and time of maximum utilization of ethanol

Dosage (μl/g mouse)	Concentration used (Vol. %)	Strain	N	Start of 5-min period showing highest rate of metabolism of $CH_3C^{14}H_2OH$ (min)	Max. rate of EtOH oxidation (μM/min/g mouse)	
4	20	DBA	2	205	0.267±0.006	0.260±0.28
		C_{57}	2	225	0.252±0.047	
1	10	DBA	11	43	0.211±0.026	0.200±0.024
		C_{57}	13	47	0.191±0.018	
0.4	4	DBA	1	20	0.186	0.164±0.031
		C_{57}	11	17	0.142	
0.2	2	DBA	3	14	0.106+0.014	0.112±0.015
		C_{57}	4	17	0.117±0.015	
0.1	1	DBA	1	10	0.080	0.078±0.003
		C_{57}	1	7	0.076	

Table XXII

Effect of buffer and pH upon the rate of oxidation of ethanol by mouse liver homogenates

Buffer	Reference	pH	Optical-density change at 340 mμ (corrected for blank)
Experiment 1[a]			
0.01 M sodium pyrophosphate	2	10	103
0.1 M glycine-NaOH	3	9.6	20
0.1 M K_2HPO_4	4	9.0	9
Experiment 2[b]			
0.01 M sodium pyrophosphate	2	8.5	53
		9.0	98
		9.4	145
		9.6	175
		10.0	211
0.06 M glycine, NaOH, and semicarbazide	5	9.2	155
		9.5	205
		9.9	261

[a]Each cell contained 3.0 ml buffer, 0.1 ml 95% ethanol, 0.1 ml DPN (10 mg/ml), and 0.1 ml of the supernatant from a liver homogenized in cold water (50 mg liver/ml) and centrifuged at 3000 rpm for 30 minutes at 0°C. Temperature during assay was 35°. The optical-density change was determined from 2 to 20 minutes.

[b]Each cell contained 3.0 ml buffer adjusted to the desired pH, 0.05 ml 20% ethanol, 0.1 ml DPN (10 mg/ml), and 0.1 ml of a mouse liver homogenate prepared as in Experiment 1. Temperature 35°, time of assay from 2 to 20 minutes.

The glycine—sodium hydroxide buffer with semicarbazide as a trapping agent, pH 9.8 to 9.9, was chosen. It was prepared daily by mixing 4 volumes 0.1 N NaOH, 7 volumes 0.1 N glycine in 0.1 N NaCl, and 1 volume semicarbazide (1.66 g semicarbazide-hydrochloride dissolved in 100 ml of 0.1 N NaOH).

The optimum DPN and ethanol concentrations were determined by using this buffer system (Table XXIII). Two mg of DPN in the final reaction mixture and 0.05 ml of 2% ethanol were chosen for the final assay conditions.

Approximately identical changes in optical density, when corrected by the appropriate blank, were obtained when a mouse liver was homogenized in either 0.25 M sucrose or in cold water. No difference was noted in activity either when the homogenate was used directly or when it was centrifuged at 3000 rpm for 30 minutes at 0° and the supernatant used. Since the uncentrifuged homogenate, being a suspension, was more opaque at 340 mμ than the centrifuged homogenate, and the blank showed a large decrease in optical density during the assay, we have used the supernatant from a mouse liver homogenized and centrifuged in cold H_2O for the assays.

Less than 3% decrease in ADH activity as determined by this assay was observed in a homogenate kept at 0° for about 3 hours. No significant loss of activity was observed on freezing and thawing the homogenate.

For routine assay of ADH activity, the mice were sacrificed in random order by decapitation and the livers were quickly removed, weighed, and homogenized in a Potter-type Teflon-pestle homogenizer (using distilled water at 0°). The final concentration of liver was 50 mg/ml. The homogenates were then centrifuged in an International refrigerated centrifuge at 0° for 30 minutes at 3000 rpm.

The reagents (3.0 ml buffer, 0.20 ml DPN solution containing 3.0 μ moles, and 0.050 ml of 2% ethanol) were placed in cuvettes and prewarmed to 35° by placing in the water-jacketed Beckman DU spectrophotometer cell compartment for 10 minutes. Then, 0.100 ml of the mouse liver homogenate supernatant was added (5 mg liver), and the difference in optical density readings at 340 mμ between 2 minutes and 20 min was determined. From the above value was subtracted the value for a blank prepared exactly as above but omitting the ethanol. The net optical density difference was converted to μmoles of ethanol oxidized per minute per gram of liver by multiplying by the factor 5.93. It was found essential not to run the blank at the same time as the run containing ethanol. If sample and blank were run simultaneously, diffusion of ethanol from sample to blank caused high and erratic blanks.

In order to determine the *in vivo* oxidation of ethanol, ethanol-1-C^{14} [7] was injected intraperitoneally into mice and the rate of excretion of $C^{14}O_2$ determined by the breath-line apparatus.[8] It was necessary to place a bubbler containing concentrated sulfuric acid in the air line prior to the ionization chamber to remove radioactive compounds (presumably ethanol) other than CO_2 in the respired air which contaminated the ionization chamber. These compounds were not completely trapped by the Anhydrone drying tube routinely used in the air line.

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[7] The 20%, 4%, and 2% ethanol solutions had a specific activity of 75 μC/ml of ethanol, the 5% and 10% ethanol solutions had a specific activity of 300 μC/ml of ethanol.

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

Table XXIII

Effect of DPN and ethanol concentration on ADH activity of mouse liver homogenates

DPN (mg)	Equivalent vol. of 2% EtOH in assay (ml)	Ethanol used in assay (μM)	Optical density change at 340 mμ (corrected for blank and for final-volume difference)
Experiment 1[a]			
0.50			196
1.0			265
2.0			330 (amount of DPN used for future assays)
4.0			384
Experiment 2[b]			
	0.50	171	285
	0.20	69	292
	0.10	34	298
	0.050	17	286 (amount of ethanol used for future assays)
	0.020	6.9	255
	0.010	3.4	212
	0.005	1.7	153

[a]Each cell contained 3.0 ml glycine-NaOH-semicarbazide buffer, pH 9.9, 0.05 ml 20% ethanol, and 0.1 ml liver homogenate prepared as described in Table XXII.

[b]Each cell contained 3.0 ml glycine-NaOH-semicarbazide buffer, pH 9.9, 0.1 ml DPN (10 mg/ml), 0.1 ml liver homogenate, and from 0.05 to 0.20 ml of an appropriately diluted ethanol solution.

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